FUNDAMENTALS OF IMMUNOLOGY

CFTRI-MYSORE
1822
Fundamentals of...

1822

1. immunity ②. antibodies
3. antigens ④ blood groups
5. complement fixation
6. antigen antibody reactions
7. anaphylaxis ⑧. allergy
9. bacteria ⑩. viruses ⑪ parasites
12. laboratory technique
13. clinical "

RNS.

FUNDAMENTALS OF IMMUNOLOGY

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1822

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PREFACE TO THE SECOND EDITION

The general character of this book, as indicated in the preface to the first edition, has not been greatly altered by the attempt to revise it and bring it up to date. The material is still addressed mainly to the same two audiences as before, namely students and research workers. For the convenience of the former group, summaries have been added to all chapters, as an aid in studying. Some errors have been corrected.

It would have been difficult, in fact impossible, to present all the new material that has accumulated which bears on the subject, especially since World War II to a great extent cut off scientific communications with foreign countries. It was never the purpose of the book to be exhaustive, but rather to present the basic facts, as is implied in the title. The appearance in the meantime of several fine reviews of parts of the field which really are specialized and complete has in any case made it less necessary or desirable to attempt to convert this book into a complete review. I refer particularly to the masterly second edition of Landsteiner's *The Specificity of Serological Reactions* (Harvard University Press, Cambridge, Mass., 1945), which fortunately was completed before Dr. Landsteiner's death, and the original, informative, stimulating treatise, *The Bacterial Cell*, by Dubos, (Harvard University Press, Cambridge, Mass., 1945). Also, both of these books have been of great assistance in my task of revision.

As before, I wish to thank the friends and colleagues who so kindly gave of their time and specialized knowledge to make the book less imperfect.

William C. Boyd

Kasr Ed-Dam
July, 1947

FROM THE PREFACE TO THE FIRST EDITION

It is the purpose of this book to serve as an introduction to immunology for medical students, chemists, biologists, and others interested in an understanding of the basic principles of the science. It is hoped that it will be of service also to those who wish to undertake research in the subject. The chief emphasis is on serology, if by that we understand the science which studies the fundamental mechanism of immunological reactions and their theoretical foundations. But the book has a slightly broader scope, for the antigenic and chemical composition of certain microorganisms and viruses and some methods of laboratory practice, and some clinical applications, are also discussed.

It is the belief of the author that, when a limited amount of time is available for a subject, as is usual in the case of immunology, it is better to spend that time in acquiring a firm grasp of the basic principles, on the assumption that application of them will then not be difficult. To attempt to study the applications first may result in a failure to grasp the underlying principles, so that each new example is approached as a separate problem, and is likely to seem much more difficult than it really is. Medical students are sometimes reproached with having "cookbook" memories, but the best cooks seem to be those who have a "feeling for the subject," rather than those who slavishly memorize a lot of recipes.

Therefore the emphasis here is on fundamentals, and at first only enough of the application is presented to illustrate these. A later chapter (XI) is devoted to immunological methods and their application to certain diseases. Here a number of theoretical and practical serological procedures are described in detail. The number of experiments probably exceeds that usually presented in the average course in immunology. By judicious selection, however, it should be possible to make up a suitable list of class experiments from this material.

In order to keep the size of the book within reasonable limits, it has been necessary to suppose that the reader has a slight previous acquaintance with organic chemistry and bacteriology. A few mathematical formulas are used in one chapter, but no acquaintance with anything more advanced than common algebra is needed. A sketch of statistical principles is given which will aid in understanding modern papers which use such methods. Study of this section and of the references given will enable workers who wish to do so to estimate the end-points (and probable significance of differences in end-points) obtained in animal experiments designed to estimate the protective power or virulence of preparations.

Although this book is intended for the beginner, it is hoped that it will also be of some use to the professional immunologist, for no attempt is made to gloss over the difficulties of the subject, or to oversimplify. Although the subject is presented without assuming any previous knowledge of it on the part of the reader, the more advanced problems are also discussed, with no attempt to avoid controversial questions. Some students may prefer books which succeed in appearing simple by being dogmatic and ignoring the opinions of those on the other side of the question, but this seems particularly dangerous in a rapidly changing subject such as ours.

One feature of this book is its almost complete abandonment of the historical method of approach, so dear to writers on immunology. To the present writer it does not seem an advantage, but rather a source of confusion, to give the reader first the concepts of past workers in the subject, only to tell him, as soon as he begins to think he has grasped these concepts, that they have been completely superseded by later ideas.

Consequently, the modern opinions on a subject, as a rule, are presented straight off. The point of view adopted here has been so far as possible that of the chemist, though no very extensive knowledge of chemistry is presupposed on the part of the reader. In general I have not tried to cite all the literature, but only the latest and most pertinent; failure to give more than one reference does not mean that no other authorities for a statement exist.

I am greatly indebted to colleagues who have read and criticized parts of the manuscript. In particular my debt to Dr. S. B. Hooker, who has read the entire manuscript and made numerous suggestions, is very great. I wish to thank also the others who generously gave their time to go over parts of the work while it was still in preparation. Especial gratitude is due to my wife, for typing the manuscript, making the index, and in many other ways assisting in the preparation of the book.

In the preparation of this book I have of course made extensive use of many books already published on the subject. I have tried to make full acknowledgement in each case in the bibliographies. I am glad to record here my particular debt to certain works, including *Traité de l'Immunité*, by Jules Bordet (Mason et Cie., Paris), *The Chemistry of Antigens and Antibodies*, by John Marrack (H. M. Stationery Office, London), *The Specificity of Serological Reactions*, by Karl Landsteiner (Charles C Thomas Co., Springfield), *Immunity: Principles and Application in Medicine and Public Health*, by Hans Zinsser, John Enders, and LeRoy Fothergill (Macmillan Co., New York), *Viruses and Virus Disease*, by Thomas Rivers (Stanford University Press); and *The Principles of Bacteriology and Immunity*, by W. W. C. Topley and G. S. Wilson (William Wood Co., Baltimore). Grateful acknowledgement is made of permission from authors and publishers to quote in several instances from the above works.

My best thanks are due also to Dr. C. A. Janeway, Dr. W. M. Stanley, Dr. J. H. Marrack, Dr. F. M. Burnet, and Dr. H. Eagle for permission to use certain illustrations and to *The Journal of Experimental Medicine* for permission to use cuts from papers which have appeared there.

William C. Boyd

Kasr Ed-Dam
November, 1942

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FUNDAMENTALS OF IMMUNOLOGY

CHAPTER I

IMMUNITY AND IMMUNOLOGY

1. BIOLOGICAL BACKGROUND

We do not know, and presumably can never know, exactly how ife began on this earth. However, we may plausibly suppose, as ther writers have done before, that life originated when the earth was still relatively young, at one or perhaps a few different points on he earth's surface. Possible modes of origin have been discussed y a number of writers, such as Wells (65a) and Oparin (47). If we ry to catalog them now, the first living forms might have been: a) complex protein molecules, (*b*) relatively simple, undifferentiated cells or blobs of protoplasm, or (*c*) some form of life we cannot imgine—but whatever they were, they were, in all probability, all alike and, in the beginning at any rate, all adapted to the same environment, the environment of the cooling earth and the warm coastal waters in which carbon dioxide and other organic compounds were o be found.

Before the newly developed organisms or complex molecules could xhibit "life" as we define it, they must have developed a means of reproduction, and thus would have been able to produce more individuals like themselves. It is not improbable that at first this was accomplished by a simple division of the enlarged cell into two new ndividuals, just as still happens among simple organisms such as some bacteria and amoebae.

Once the process of reproduction had begun, a new phenomenon, that of *competition*, would exhibit itself; for an organism capable of reproduction must necessarily be able to reproduce itself at a higher rate than the mean rate which is requisite just to preserve constancy of numbers over a long period of time. This is clear, since climatic and other conditions may vary, the supply of food may diminish, or other unfavorable conditions may arise, any one of which may drastically reduce the numbers of a population. Unless the population has a capacity to recoup its losses and make good its diminished numers, it is only a matter of time until it will be wiped out. Since the hypothetical organisms from which all life that we know is supposed to be descended did not die out, they doubtless were capable of reproduction on a more rapid scale than was needed merely to preserve the same number of individuals from year to year, or from generation to generation. But, as soon as larger numbers of individuals are produced, the phenomenon of competition begins, because the food supply, the supply of substances which the young organism may assimilate and turn to its own uses, is finite on a finite planet; and, besides, it is unlikely that the first living organisms would have been able to make use of, or even reach, the food of more than a local area of the earth's surface. Before long there will be more organisms than there is food supply to support them, and competition among the organisms for the food supply will begin. Or competition merely for space in which to live may be a factor.

As soon as competition begins, we may suppose that evolution has also begun; for competition between organisms, coupled with the fact that organisms occasionally vary in an inheritable way from the ancestral type, bring evolution about. As a result of evolution, new or more complex organisms may be produced; or the new organisms may perhaps be equally simple but capable of assimilating a slightly different type of foodstuff, or may have other modifications. Our archaeological record indicates that life, originating doubtless in shallow waters adjacent to the land, gradually spread through the waters and eventually emerged onto the dry land, finally producing, through evolution, the terrestrial forms with which we are all familiar.

As soon as different forms or species were produced, there would begin, in addition to the competition which had previously existed between different individuals all of the same kind for food supplies, competition between individuals of different species. Each organism would have to take part in what scientists of the nineteenth century called the "struggle for life." Biologists have shown that this struggle for life is indeed a much more bitter and relentless one than a casual examination of the situation might lead one to suppose. No holds are barred, and no slight adaptive change or useful device which makes for survival will be overlooked in the long run, provided it is within the scope of the possibilities of living matter.

There are a number of ways in which different organisms and different species may compete with each other. In the first place, they may simply want to occupy the same space; in other words, to live on the same half-acre of land or in the same cubic centimeter of sea water. If there is room there for only so much living matter, only a limited number of individuals will be able to occupy this location. Second, and usually a more crucial limitation, is the finite character of the food supply. In the case of plant life, the food supply consists of carbon dioxide from the air, plus minerals and organic matter in the soil. Any amateur gardener who has observed the way an undesirable sort of grass may take over a whole area formerly occupied by some other plant, considered useful or ornamental by man, will realize the intensity of the struggle for food supply and the completeness with which one variety of living organism may gain ascendency over the other. In an area where formerly hundreds or perhaps thousands of individuals per square yard of a certain species could be found, five years later not a single individual can be discovered.

At some stage of evolution, one or more species must have made the momentous discovery that a very convenient source of food consists of the tissues of other organisms. Perhaps dead organisms were utilized at first, but it would be but a short step to the consumption of living organisms. The whole of the animal kingdom depends upon eating plant life in some form or other—directly or indirectly—and thus obtains the protein which it needs to build up its tissues, and the carbohydrate or fat to provide energy for its activity. It is only the green plants and certain other vegetable organisms which can synthesize these substances from inorganic materials and carbon dioxide.

Once the importance and desirability of eating the other fellow was discovered, evolution had the opportunity to take a further step, whereby an animal accustomed to eating plant life could also develop the taste for eating other animals. The new taste might eventually supplant the old vegetarian habits completely. A lion eats no plant life, but nevertheless lives on plants only at one remove, since its usual food consists of large herbivorous mammals. (Of course we do not mean to imply that evolution had to wait for the animals as we now know them to be differentiated to develop this step of one form's eating other or like forms.)

Competition between the carnivorous animals also developed, and their numbers in turn are limited by the available food supply and by the varying abilities which evolution has conferred upon them to obtain it. The numbers of the carnivores may be indirectly an index of the fertility of the soil of a region. If there are not many herbivorous animals in a particular locality, there will not be many lions.

It was probably not very late in the history of evolution that another and about equally important discovery was made. Instead of simply eating other individuals and thus obtaining nourishment, certain organisms can learn to live with or upon other organisms and obtain their nourishment, either by eating portions of the tissues or by absorbing food which would otherwise be utilized by the host. This may or may not result in the death of the host. Organisms that live upon another organism and depend upon it for a source of nourishment are known as parasites. Evolution has operated on the parasite as well as upon other living organisms, and the world as we know it contains many examples of extremely accurate adaptations of the parasite to its host. The parasite may in many cases become smaller, and thus less conspicuous, and better able to find lodgment in the tissues of the host. It may become, in some respects, simpler—morphologically or chemically—thereby undergoing a process which we usually refer to as degeneration.

While parasites were developing, however, the organisms which they made their prey were, in the evolutionary sense, not idle themselves. Whatever the first forms of life on earth may have been, we find at the present time that all organisms have provided themselves with some membrane or sheath to protect themselves against invasion from the outside. In many cases, skin and various kinds of armor have also been developed. The porcupine has developed a coat of quills which must be very effective in preventing any carnivorous animal from eating him, judging by the results when a dog attempts to attack a porcupine. Quills and other defensive armor would be quite effective against large predatory animals but less effective against smaller organisms which had become parasites, and which either were from the beginning or had become so small as to be invisible to the eye. Such organisms would be able to find their way into the tissues of the host either through natural openings of the body or by actually boring a path, as is done by the free swimming form of certain parasites, such as *Schistosoma*. This process has gone on to such an extent that it may be safely said that probably no organism of any considerable complexity exists which is not subject, at some stage of its existence, to the attack of some sort of parasite or microorganism.

There are two classes of parasitic organisms of predominant concern to us, which we call bacteria and viruses. Some think the latter may have evolved by degeneration from the former. The nature of these forms of life will be discussed later on in this book. Suffice it to say here that they are small organisms, invisible to the eye without artificial aid, which may on occasion invade the tissues of suitable hosts. When such an invasion occurs, we speak of it as an infection.

Now just as organisms develop protective devices such as spines or armor to protect themselves against being eaten, so also have they, in practically all cases that we know about, developed protective devices against parasites, including the tiny organisms called bacteria and viruses. Some of these protective devices consist of relatively impermeable membranes, such as skin and the mucous membranes of the higher animals. Others include, apparently, the ability to alter the body temperature to one unsuitable to the would-be invader. There must be a great many other such devices. For, just as the parasite is capable, in the course of evolution, of evolving in a manner so as to be able to invade a different host, or more effectively to invade the same host, the host is capable of evolving in such a way that it can resist invasion. The production by artificial selection of plant resistance to various plant diseases such as the rust disease of wheat, etc., is but an imitation of this natural process.

Furthermore, another somewhat accidental factor must operate. In the course of adapting to a certain host, a parasite may automatically lose its adaptation to other hosts, for the adaptation required may involve some physical or chemical change, such as the alteration of one of its enzymes to make it specifically capable of hydrolyzing some particular compound found in the tissues of the desired host. The new enzyme may not work so well against chemical substances found in other organisms. Or a parasite may lose, in the course of evolution, the capacity to produce some vitamin or other substance essential to its metabolism, because it finds this substance in the tissues of the particular host or hosts it is currently living on. Changes of this sort result in specialization. But such changes may deprive the parasite of certain more general abilities which enabled it to invade, though perhaps less successfully, a number of different hosts. It may become completely specialized to a single host, and then, should it find this particular host unavailable, it will die. Organisms to which it is not adapted will be, as we say, immune to attacks of this parasite—that is, they will not be affected by it. This is the correct use of the word "immune," which in its original meaning implies complete freedom or exemption from some certain thing. Thus, pigs are immune to smallpox, and the rat seems to be absolutely immune to diphtheria.

But we shall seldom use the word immune in the strict sense here.

We are going to be interested, not in absolute immunity, but in degrees of refractoriness to infection, and the use of the word "immunity" in this way has become accepted terminology. Immunity is only one of the weapons in the basic struggle for life, and denotes the *resistance* which each organism offers against aggression by another. Study of immunity, in the sense in which we are going to use the word, can be seen from the above paragraphs to be only a branch of biology, or in a narrower sense only a branch of ecology.

An interesting treatment of disease from the point of view of the biologist is seen in the book by Burnet (7). Burnet points out that there is good reason to think that, even within historical times, there have been changes in certain human diseases, which represent an adaptation either of the host to the parasite, or of the parasite to the host, or perhaps both. He points out that in the majority of cases, the best adaptation has been reached when the parasite causes the host comparatively little damage and does not kill it, but is able to perpetuate itself by passing from one host to another, either directly or indirectly. From the point of view of the parasite, of course, the object is to keep the amount of living matter, organized in its own particular way, as great as possible. The organization of the molecules in a particle of influenza virus, for example, is different from the organization of the molecules in one of the cells of *Homo sapiens*. Burnet considers that diseases like herpes simplex (cold sores or fever blisters), which cause the host very little trouble, and in which the virus seems to persist from year to year for long periods of time, represent about the optimum adaptation of the parasite to the host. He considers poliomyelitis, a disease which seems to have been diminishing in severity in recent times, to be an example of a disease in the process of evolution (7a).

2. IMMUNITY

As we have just pointed out, the subject we propose to study here is not covered by the word immunity in its original meaning. What we are really interested in is degrees of resistance to disease, and particularly in how we may increase inadequate resistance artificially (29, 63). The study of the procedures we use and of the mechanisms by which resistance is increased is usually called *immunology*. The study of the diagnostic and experimental procedures connected with this problem is usually called *serology*, because they involve the use of serum. Other subjects, such as blood grouping and forensic precipitin tests, are included under immunology although, for example, the existence of blood groups in man seems to be, on the whole, a perfectly normal phenomenon without relation to any disease. But the procedures used, and the mechanisms involved, are essentially the same as those developed in research on immunity.

The study of the different ways in which an organism resists the attack of a parasite, including the study of the *natural resistance* the organism has before the attack is begun, is extremely interesting. The reader will find practically all of our present day knowledge on the latter subject summarized in a book by Perla and Marmorston (51). Even this book probably falls far short of a complete coverage of all the natural mechanisms of resistance to infection.

Some of the resistance which an organism offers to infections does not seem to be directed against any particular microorganism or even any particular group of organisms, and we thus call it *nonspecific*. Nonspecific resistance undoubtedly plays a large role in immunity and it is likely that our knowledge of its importance is still very incomplete. Many observers (24, 25, 43, 44, 53) have reported that local treatment with certain agents, even nonspecific irritants, which provoke inflammation, will increase the resistance in general.

3. INFLAMMATION

Inflammation is a fundamental phenomenon occurring in higher animals (43). It involves lymphatic structures, vascular channels, and the locally affected tissues. It is initiated by a disturbance in fluid exchange, which markedly deranges the normal capillary circulation. One of the major changes is an increase in capillary permeability. This is readily demonstrable by the local seepage of material deliberately introduced into the circulation. Diazo dyes, ferric chloride, graphite particles, and bacteria will readily concentrate from the circulation into acutely inflamed foci (45).

Menkin (42, 43) believes that there is liberated into exudates in inflamed areas a substance, not directly related to histamine, which is thermostable and diffusable through a cellophane membrane. (In immunology, "thermostable" means resistant to heating at 55-60°C. for 30–60 minutes.) It seems to be a peptide, but a prosthetic group may be attached. This peptide-like substance, which has been called *leukotaxine*, not only increases capillary permeability, but also induces the migration of polymorphonuclear leucocytes through the capillary wall.

Increased capillary permeability allows the free passage of plasma proteins from the circulation into the extracapillary spaces. Fibrinogen is converted to fibrin with the release of thrombokinase following

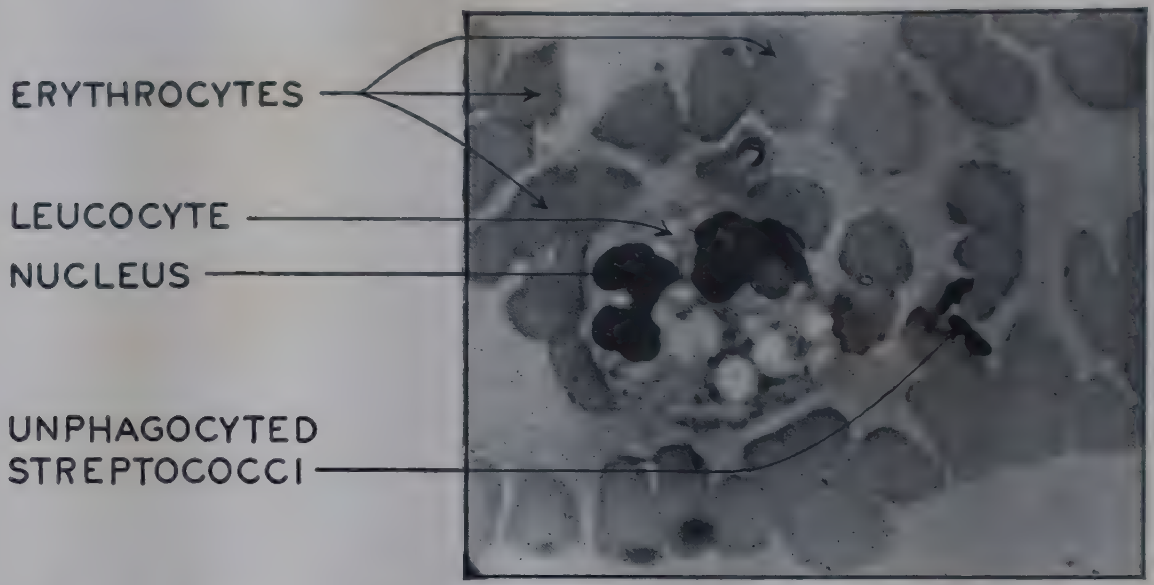
ERYTHROCYTES
LEUCOCYTE
NUCLEUS
UNPHAGOCYTED STREPTOCOCCI

Fig. 1a. Smear of defibrinated blood from patient with puerperal sepsis due to hemolytic streptococci, mixed with drop of young culture of streptococci (*courtesy of Dr. Charles Janeway*).

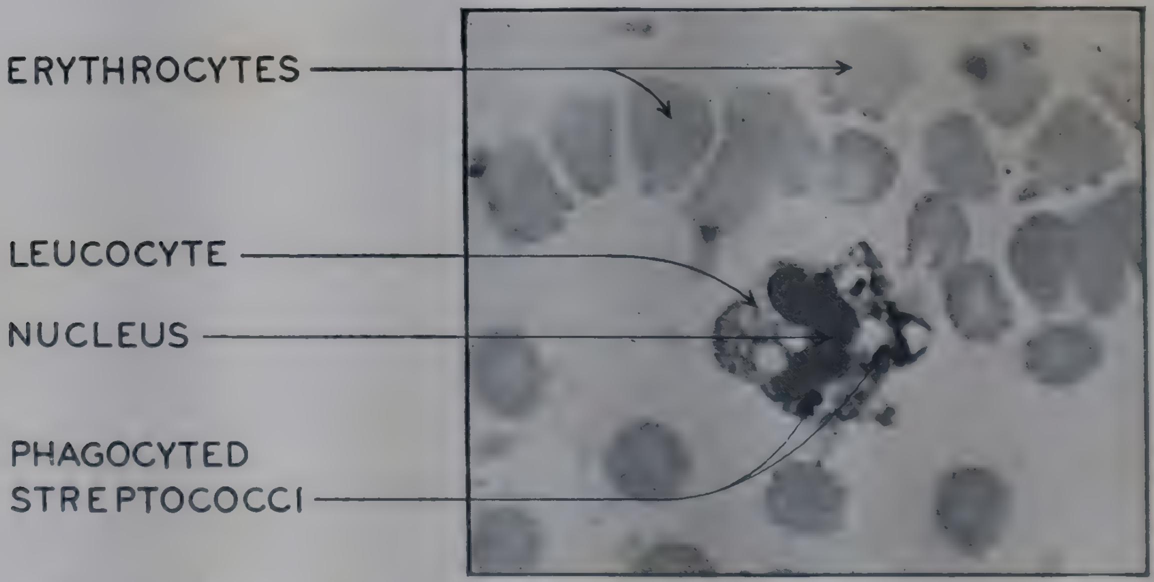
ERYTHROCYTES
LEUCOCYTE
NUCLEUS
PHAGOCYTED STREPTOCOCCI

Fig. 1b. Preparation as above, plus a drop of serum from an immune person (*courtesy of Dr. Charles Janeway*). Phagocyted streptococci seen within leucocyte. Note absence of capsules on streptococci.

injury to cellular structures. A network of fibrin is formed in tissue distended by edema, and lymphatic channels become plugged with fibrin, thus inducing a lymphatic blockade. This tends to prevent the dissemination of infectious material or microorganisms from the focus of inflammation. The migration of leucocytes into the area serves further to wall off the invading microorganisms, which will be dealt with, if the infection does not spread further, by phagocytosis (see below).

A protein-like *leucocytosis-promoting factor* is also liberated from injured cells. It not only induces a discharge of immature leucocytes from the bone marrow, but also causes marked hyperplasia of elements of the bone marrow.

Inflammation is a manifestation of severe cellular injury. The injury seems partly due to the liberation of another substance in the exudate which has been termed necrosin. Menkin (42) has outlined the whole process as follows:

(*1*) Disturbance in local fluid exchange.
  (*a*) Increase in capillary permeability.
  (*b*) Initial increase in lymph flow.
(*2*) Localization of the irritant (fixation).
(*3*) Migration of the leucocytes.
  (*a*) Influx of polymorphonuclear leucocytes.
  (*b*) Other cytological changes dependent on the local *p*H.
  (*c*) Leucocytosis in the circulation.

As a result of this sequence of events, the localization and ultimate disposal of the irritant is favored. If this is accomplished, repair of the injured area begins.

It is not possible to measure accurately the extent of resistance which an animal may develop locally as the result of inflammation, although Menkin has experimentally used the degree of retention of a dye as a rough measure of the retentive efficiency of the inflammatory process.

4. PHAGOCYTOSIS

An amoeba gets its food simply by engulfing particles of foreign substance and absorbing the useful portion. An amoeba can deal thus with living microorganisms, and in so doing it thereby provides itself with a most effective defense against them. It might be suspected that such a handy form of defense would not be totally lost in the course of evolution, and in fact in the 1880's Metchnikoff discovered that within the more complicated organisms there exist simple cells capable of ingesting and disposing of particulate matter. This phenomenon is called *phagocytosis* and represents the great clearing mechanism of the blood (Fig. 1).

There is abundant evidence that the activity of some free and fixed cells in the blood and tissues is an important part of the mechanism of immunity.

When dyes and suspensions are injected intravenously into an animal, quite often they are not eliminated in the urine or bile (as are for instance phenolphthalein and its derivatives), but are deposited in various organs such as the spleen, liver, lymph nodes, and bone marrow, and they may remain there in recognizable form for weeks. For a review of the literature and of the newer experiments see Cappell (11, 12).

Investigation has shown that these dyes are stored inside certain cells which the histologist calls *histiocytes*, but some immunologists, following Metchnikoff, call them *macrophages*. These cells have a characteristic distribution throughout the body, and may be divided into two main types: sessile, and wandering. The most active of the sessile (fixed) histiocytes are found in specialized areas of the vascular or lymphatic endothelium. Chiefly important are the endothelial cells of the capillaries of the liver (Kupffer cells), of the sinuses of the spleen, of the venous sinusoids of the bone marrow, of the capillaries and medullary sinusoids of the adrenals, of the capillaries of the pituitary, and of the sinuses of the various lymph glands of the body. The reticulocytes, which are disposed about the reticulum fibers in the interstices of the tissues, are somewhat less active. Wandering histiocytes occur in all the tissue spaces and to some extent in the blood, especially in vessels in internal organs.

The similarities in behavior of these cells have led to their being classified together, as a system of cells all with the same special function, in spite of their wide separation in the body. Aschoff's classification (4, 5) is summarized in the accompanying table.

Reticulo-endothelial System

Sessile histiocytes (sessile macrophages)		*Wandering histiocytes* (wandering macrophages)	
Less active	Very active		
Reticular cells of spleen lymphatic glands and tissues thymus	*Endothelium of* liver capillaries (Kupffer cells) spleen sinuses lymph sinuses marrow sinuses adrenal capillaries pituitary capillaries	*Tissue histiocytes* (tissue macrophages)	*Blood histiocytes* (blood macrophages)

There may be some division of labor in the reticulo-endothelial system, in that some parts may be more active in removing aged or fragmentary red corpuscles or leucocytes, while others tend to specialize more in removing bacteria or other smaller particles. Species differences are also found in the relative activity of the various regions. It will be seen later that species differences are also found in the behavior of organs with respect to anaphylaxis and to antibody producing power.

There is in addition another class of phagocytic cells, the polymorphonuclear leucocytes of the blood. They do not take up "vital" stains in the same way as the cells of the reticulo-endothelial system, but under certain conditions do actively remove particulate material from the circulation. These are the *microphages* of Metchnikoff. The neutrophiles are active, and the basophiles slightly active; there are conflicting reports as to the activity of the eosinophiles.

It is now no longer doubted that this same mechanism which has been observed to free the blood stream from inert particles is also the chief factor in freeing it from bacteria. If bacteria are injected into the blood stream, they disappear rapidly from the circulation and are found to be taken up by the reticulo-endothelial cells, especially those of the liver, spleen, and bone marrow.

Mechanism of Phagocytosis. The wandering cells are doubtless brought into contact with particles in the circulation by collisions. But phagocytes can also crawl by amoeboid motions, and it has been found that they are positively attracted to living and dead bacteria and to certain kinds of inert particles. This is called chemotropism, or chemotaxis.

Ingenious and plausible attempts have been made (20, 21, 46) to account for the ingestion of particles by the phagocyte in physicochemical terms. Mudd, McCutcheon, and Lucké (46) present interesting evidence to support this interpretation. Figure 2, taken from their paper, shows the parallelism between surface changes and phagocytosis. The avian tubercle bacillus was exposed to the action of homologous immune serum and to various protein fractions separated from the serum; the bacillus was then washed and exposed to phagocytes, with the results shown.

It thus appears that there are substances in the immune serum which have the property of increasing the degree of phagocytosis of the corresponding bacteria. Normal serum also has some, but less, activity.

Before investigating the nature of the substances in serum which favor phagocytosis, we may pause to ask if the phagocytes themselves are altered in any way as an animal becomes immune (see 37). Kahn (37) and Lurie (39) evidently believe they are, and Metchnikoff, although he recognised the "stimulating" effect of serum, con-

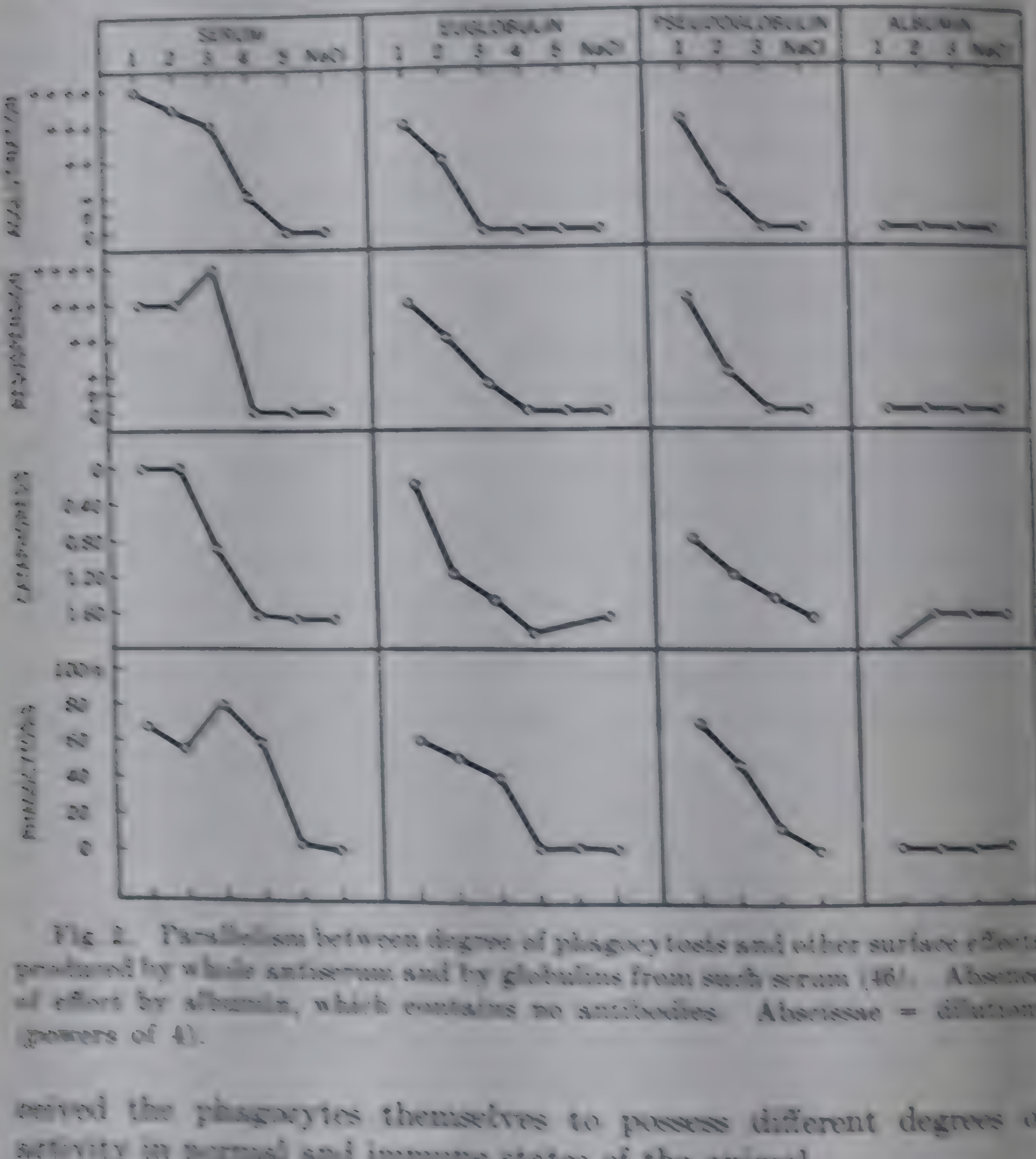

Fig. 2. Parallelism between degree of phagocytosis and other surface effects produced by whole antiserum and by globulins from such serum (46). Absence of effect by albumin, which contains no antibodies. Abscissae = dilutions (powers of 4).

ceived the phagocytes themselves to possess different degrees of activity in normal and immune states of the animal.

Not only is the local mobilization of leucocytes one of the mechanisms of immunity, but there also seems to be an increased production of leucocytes. Leucocytosis, as it is called, following an injection of microorganisms or foreign cells is especially marked in immune animals; it can also be provoked to a less degree by nonspecific stimuli, such as the injection of broth, peptone, and certain vegetable toxins.

"Opsonic" Action of the Serum. It was found by Denys and Leclef (17) that the destruction of bacteria by phagocytosis was powerfully increased when immune serum was added to the leucocytes of either a normal or an immunized animal (see Figures 1 and 2). This was confirmed by later workers, and since then investigations of phagocytosis have concentrated chiefly on the role of the serum.

The antibodies facilitating phagocytosis of bacteria were called "bacteriotropins." The word "opsonins" (from *ὀψώνης*, a purveyor of fish or dainties) was coined by Wright and Douglas for these then purely hypothetical substances.

It has been suggested that the opsonins of serum consist of two components: thermostable (see page 7) antibody and a thermolabile substance similar to a component of complement (see Chapter VII). Both of these appear to be found also in normal serum, but the antibody concentration is greater in immune serum. Antibody alone will opsonize but not quite as well (see Fig. 3).

The antibody involved is in many cases doubtless the same antibody which under other circumstances is observed (see Chapter II) to agglutinate, and fix hemolytic complement. The presence of this antibody on the surface evidently produces the change in surface conditions of the organisms which opsonizes.

The function of the thermolabile factor resembling complement is not fully understood, but one possible function is that of a catalyst which increases the speed of combination between antibody and antigen particles. It will be seen in Figure 3 that the ultimate opsonization without complement is about the same as with it, but that without complement this stage is reached more slowly.

The thermolabile substance is closely related to complement, and some portions of it may perhaps be the same, but there are certain differences which do not allow us to equate them (30, 31, 66).

Importance of Phagocytosis. Phagocytosis is of decisive importance in the animal's resistance to many diseases. In spite of the fact that the cooperation, so to speak, of serum antibody is required, in some, perhaps most, diseases, recovery would be impossible without phagocytosis. The general importance of the phenomenon has been stated by Bordet (6) to be shown by:

(*a*) The strict parallelism between the vigor of phagocytosis and the degree of resistance. There seem to be no examples of recovery without the intervention of phagocytes.

(*b*) The sterilizing power of the body fluids for many pathogenic bacteria is negligible; even when raised by infection it is not sufficient to explain the complete destruction of the relatively large numbers of microorganisms which can be taken care of by the animal.

(*c*) The natural or acquired properties to which microorganisms owe their virulence are essentially antiphagocytic mechanisms. Harmless organisms are easily captured and destroyed by phagocytosis; dangerous organisms resist, indeed it is because they resist that they are dangerous.

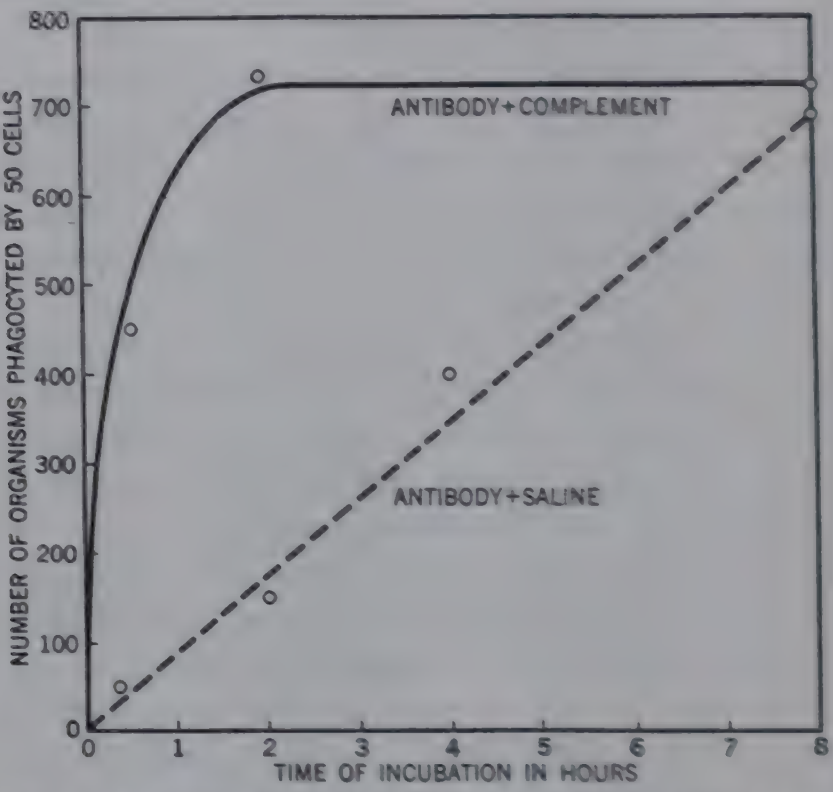

Fig. 3. Phagocytosis with and without complement (67).

(*d*) Inoculation of pathogenic microorganisms is less dangerous when it is made into a region rich in phagocytes. Thus inoculation of pigeons, normally refractory to anthrax, by injecting the bacilli into the aqueous humor of the eye where leucocytes are mobilized only slowly, will produce the disease. The longer the bacteria have to multiply before leucocytic response, the more serious the infection; also in this time the invading organisms may be able to make protective adaptations. Preliminary treatment tending to mobilize leucocytes increases resistance to infection. It has even been claimed that injection of leucocytes or leucocyte extracts has protective and curative power.

(*e*) Any influence that interferes with phagocytosis diminishes resistance proportionally. Tetanus spores are easily phagocyted, but concomitant injection of substances toxic for leucocytes, such as lactic acid or quinine, favors infection. Even sterile, inert material may make the spores more dangerous by distracting the phagocytes from the real struggle. It is also apparent from medical and surgical experience that wounds and bruises, as well as cold, starvation, and poor general condition, favor infection.

(*f*) Very generally a favorable turn of an infection is accompanied by an increase in the number of leucocytes. Immunized animals generally show a leucocytosis, which persists for a long time.

Fate of Bacteria Ingested by Phagocytes. It is natural to suppose that as soon as a microorganism is ingested by a phagocyte it is already dead; and it is certainly sometimes killed (52), but this is not always the case. Indications have been found that in some cases in susceptible animals pathogenic microorganisms remain viable for some time after being taken up by the phagocytes. Rous and Jones (57) showed that phagocyted typhoid bacilli survived in the cells and were protected from the action of lytic antibody. Fothergill, Chandler, and Dingle (23) found evidence suggesting that ingested influenza bacilli remain viable. Work by Lyons (40) shows that toxigenic strains of staphylococci are not killed by the leucocytes which ingest them. Some virulent bacteria seem to be liberated again after ingestion, perhaps after they have killed the phagocytes (see below). These are exceptions to the general rule, however, and the great mass of data shows that phagocytes can and often do kill ingested organisms, and in many cases the swelling, granulation, fragmentation, and lysis can easily be observed microscopically. Pneumococci have been observed to be phagocyted and destroyed. Metchnikoff found that, in animals naturally resistant to anthrax, phagocytosis occurred promptly and all the ingested bacilli were engulfed by leucocytes, while almost no phagocytosis occurred in susceptible animals. A number of workers, such as Ørskov and collaborators (41), Cannon and co-workers (10, 16), have shown by actual cultivation experiments, using for example ground-up tissues from the animals, that ingestion of the bacteria actually resulted in death of the bacteria. Also, numerous test tube experiments show that leucocytes kill bacteria. Some organisms are practically completely resistant to the action of serum, even if complement is present, but nevertheless succumb to phagocytosis (18, 26).

Resistance of Bacteria to Phagocytosis. In many cases it has been found that virulent forms of bacteria are more resistant to phagocytosis; there are various factors causing this. The type-specific polysaccharides in the capsule of the pneumococcus (see Chapter IV) seem to help defend the organism against phagocytosis. In themselves they are not attractive to leucocytes, and apparently they are not harmful. But a pneumococcus will not be phagocyted until antibody combines with it and renders the surface "attractive" to phagocytes. Since the polysaccharides are continually being cast off into the surrounding fluids in soluble form, and since they have been shown to combine with antibody which would otherwise coat the bacterial surface, they thereby render this antibody unavailable for defense. In other organisms a relation between susceptibility to phagocytosis and possession in the cell body of a certain substance (antigen) has been shown. An example is the "Vi antigen" of Felix, discussed on page 155.

The rather "immunological" suggestion has been made that the aggressive action of bacteria is aided by their production of specific substances termed *aggressins*. The effect is probably a fact; but it is doubtful if there always exists a particular substance to account for it. In pneumonia, however, it may be that the soluble capsular polysaccharide plays such a role (38). A specific antiopsonic action (19) has apparently been found in some instances.

A number of microorganisms, including streptococci, staphylococci, and pneumococci, produce substances having a destructive action on leucocytes. These substances are called *leucocidins*. They evidently aid the bacteria in their struggle with the leucocytes.

It has been shown that venoms (19a, 19b) and certain pathogenic bacteria (12a, 45b, 45c, 56a) contain a "spreading factor" (33a, 40a) which has been identified with the enzyme hyaluronidase (32b, 40b), which causes depolymerization of a mucopolysaccharide, hyaluronic acid (45a), which is found in the skin. According to Duran-Reynals (19c) the degree of invasiveness of bacteria is largely determined by the amount of the spreading factor present, and the successive phases of infection induced by invasive bacteria include (*a*) the hydrolysis of the mucoid ground substance of the connective tissue, which otherwise acts as a physiological obstacle to the spread of the infection, and (*b*) spreading primarily through the interstitial system of the connective tissue. Hyaluronidase could therefore well be called "invasin."

Haas (32a) considers that he has demonstrated in plasmas of mammals, birds, and fish a counter-enzyme, which he calls antivasin I. Bacteria and venoms, however, contain an enzyme proinvasin I, which rapidly inactivates antivasin I. A second protective enzyme, antivasin II is reported by Haas to be present in normal plasma. It is thought to act by destroying provasin I. Other bacterial and plasma enzymes are hypothecated. It would seem that an important clue to part at least of the mechanism of nonspecific resistance to infection has been found.

Measurement of Phagocytic Power. Since phagocytic power is a useful index of the degree of resistance of the animal to infection, methods of measurement are important. Unfortunately the technic is difficult, and it is difficult to get absolutely reliable results (see Chapter XI). For this reason measurement of antibody content seems likely to remain the easier method of estimating immunity and is the one usually used.

5. Kinds of Immunity

After successfully resisting invasion of a parasite or group of parasites, a host is likely to have, in addition to whatever natural immunity it may have started with (which we may assume stays about the same), a higher degree of immunity than it had originally. This increased immunity is a result of contact with the parasite, and is thus an acquired immunity. At least two senses of the word immunity, even in our usage of it, are therefore called for.

Let us first consider the kind of immunity called innate or constitutional immunity, which is independent of previous contacts with the parasite. There is a popular idea that susceptibility to certain diseases runs in families, and that some ethnic groups of the human race are more susceptible to some ailments than are other peoples. When such beliefs are examined scientifically they are not always confirmed, but apparently it is no longer doubted that some racial and hereditary differences in susceptibility exist. The greater resistance to anthrax of Algerian sheep, as compared with European sheep, is well established, and some breeds of mice are much more susceptible than others to some infections. The mechanisms of natural immunity, aside from inflammation and phagocytosis, are not well understood. Possible factors are alterations in the permeability of the skin and mucosa. Some consider the Shwartzman phenomenon, discussed in Chapter IX, to have a bearing on natural resistance. Webster and his associates (65) succeeded in breeding strains of mice either more or less susceptible even to inorganic poisons than the stock strain of the Rockefeller Institute.

Racial differences in susceptibility in man probably also exist, but are hard to verify because of the complexity of the problem, and in many cases the explanation of the apparent differences is really some differences in habits or living conditions or immunity acquired from mild infections in childhood. It seems likely, however, that some aboriginal peoples were much more susceptible than Europeans to introduced diseases such as measles and tuberculosis. Also, Russell and Salmon (58) have presented evidence that the Welsh are racially more susceptible to tuberculosis than are the English. The data of Hopkins (34) suggest a hereditary factor in susceptibility to leprosy. Very little is known of the hereditary mechanism of differences in innate resistance (see 51).

The second kind of immunity, and the one chiefly to be studied here, is acquired immunity. This again falls naturally into two classes, active, and passive. Active immunity includes that resulting from recovery from infection or artificial inoculation; it is called active, since it is considered that the tissues of the animal play an active part in producing it.

Passive immunity is transmitted to the animal, either naturally from the mother through the placenta or in the colostrum, or artificially by injection of serum or other proteins derived from an immune animal.

This gives us the following classification of kinds of immunity; it is logical but not very informative.

(*I*) Innate immunity (constitutional or racial).
(*II*) Acquired immunity.
  (*1*) Active: (*a*) natural; (*b*) artificial.
  (*2*) Passive: (*a*) natural (congenital); (*b*) artificial.

We shall on the whole be more interested here in active than in passive immunity.

6. Grades of Immunity

Immunity, in our sense of the word, can exist in varying degrees. It is rather rare to find an animal absolutely without resistance to an infection often observed in the species. In the case of common fatal infections, this is obvious, for such an animal, inevitably contracting the disease, would already have succumbed before we had the opportunity to observe him. In the case of some diseases with which the ordinary individual has very little contact, such as psittacosis and tularemia, the resistance of the average person is very low, and at first almost every laboratory worker who worked with these diseases contracted them.

The infectiousness of any given disease depends, of course, not only on the host but also on the invading organism. Some diseases are enormously more infectious than others, and some are much more serious when contracted. We shall arbitrarily neglect the interesting question of intrinsic virulence in various organisms and strains of organisms, considering it a branch of bacteriology and not of immunology.

Topley and Wilson (63) have classified grades of immunity (to bacterial diseases) as follows (slightly modified):

(*1*) Completely susceptible. (All or almost all infected individuals will develop fatal bacteremic infections. Local lesions infrequent and minimal.)

(*2*) Low-grade immunity. (Fatal bacteremic infections less common. Local lesions more frequent and more pronounced. A small but increasing number of latent infections.)

(*3*) Medium immunity. (Bacteremia and death much less frequent. Local lesions common and relatively extensive. Latent infections more frequent.)

(*4*) High-grade immunity. (No deaths, bacteremia infrequent and when found slight and transient. Local lesions less frequent and when found less extensive. Latent infections most frequent or on decline.)

(*5*) Complete immunity (if it exists). (The animal is completely resistant to all attacks of the infectious agent.)

In prophylactic treatment of human beings, we are often satisfied if we can achieve immunity of grades *3* or *4*.

7. MEASUREMENT OF IMMUNITY

A number of immunological procedures are directed towards the end of measuring resistance, and these will be discussed in the appropriate place. Here a few general remarks may be made.

As a rule it is difficult or impossible to measure the resistance of an individual animal. For suppose we give a measured dose of the active disease-producing agent, or of some toxic product of the agent, and observe what happens. If the animal dies, its resistance was less than that of animals which can tolerate this dose. But how much less? To this question we have no answer, nor can we obtain one, for the animal is now gone. If we start with a smaller dose, which the animal successfully resists, we can not proceed to determine its resistance precisely by trying successively larger doses, since the first dose will have altered the resistance which we wanted to measure.

A possible way out of this dilemma consists in testing simultaneously a large number of individuals, all with the same history, employing different doses. Then from the different numbers which succumb to each dose, we can estimate, subject to some uncertainty, the average degree of resistance. This matter will be discussed under a more appropriate heading in Chapter XI.

With human beings we often wish we could actually determine the degree of resistance of an individual, so that we may know if he is susceptible to a given disease, and thus needs prophylactic immunization (if this is in our power), or ought to be warned to avoid sources of infection. It would also be desirable to be able to give the individual an estimate of the degree of his susceptibility. If we knew the complete mechanism of immunity and could measure in a given person all the factors which were concerned, our information might make it possible to give this sort of individual diagnosis of the degree of immunity.

Suppose, for example, that it was known that immunity to a certain disease was due *solely* to the increase in amount of some one well characterized protein constituent in the blood. If we could measure the concentration of this protein accurately, we could make determinations for it on groups of individuals and then correlate this information with their average resistance. From the results we could say that a certain level of this protein constituent in the blood (allowing perhaps for some random variation) meant a certain degree of immunity. This test could be performed on any individual and it would be possible to predict with considerable accuracy whether or not he would succumb to infection, and perhaps even how severe an attack he would have, assuming we could predict he would eventually recover.

Actually the situation, as will be seen below, is mostly not so simple, but there are some cases of a slightly different nature where analogous methods can really be applied. In the standardization of type I antipneumococcal sera, Heidelberger, Sia, and Kendall (33) showed that a close parallel existed in these sera between the amount of protein specifically precipitable by the type I specific pneumococcal polysaccharide and the number of mouse protection units. Since only three mice were used for each dilution of serum, it is entirely conceivable that in this experiment (see Chapter XI) the nitrogen determinations actually gave a better index of the power of the serum than did the numerical results of the mouse tests (see 28). Skin tests (e.g., the Schick test, Chapter X) can also sometimes give information as to the state of immunity of the individual.

8. Mechanisms of Acquired Immunity

There seem to be four ways in which *increased resistance* to a disease can manifest itself in an animal. (*a*) There may be some increase in the nonspecific factors of resistance, such as a decrease in the permeability of the skin and mucosa. (*b*) The tissues may be altered in such a way that they become intrinsically resistant. (*c*) Mobile cellular elements (phagocytes) may be made available more quickly to fight off the invading organisms. (*d*) Soluble substances which tend to protect against the infection (antibodies) may be secreted into the blood and tissue fluids.

Tissue Immunity

By tissue immunity we mean an alteration in the tissues themselves, aside from (*c*) the mobilization of cellular defenses in the blood and fluids, and (*d*) the production of soluble defense substances such as antibodies, which makes them more resistant to a specific infection. Not all workers are agreed as to the existence of such a type of immunity; others have written whole books on the subject.

A priori, it would seem entirely possible that tissue immunity, as defined above, and as distinct from complete tissue insusceptibility (page 19), could exist; but there is not much evidence that it does. However, there are some observations suggesting that something other than antibodies is operative in immunity. For one thing, in some diseases a large amount of antibody may be present, as in typhoid fever, without any noticeable effect. Immunity to certain other diseases may persist for years after all detectable traces of antibody have disappeared.

The course of erysipelas in human beings might suggest that the parts of the skin which have recovered from the infection may be temporarily resistant, while adjacent parts are still susceptible. Similar conclusions are indicated by the work of Amoss and Bliss (1), Cobbett and Melsome (13, 14), Gay and Rhodes (27), and Rivers (56).

On the other hand, Cannon and Sullivan (9), and Walsh, Sullivan, and Cannon (64) found evidence that certain parts of animals which had experimentally been brought directly in contact with *Salmonella schottmuelleri* tended to have a greater concentration of antibodies than even the spleen and liver, suggesting perhaps that at least part of the local resistance was due to antibodies.

Zinsser, Enders, and Fothergill (67) consider that the available evidence fails to demonstrate "a new and heretofore unrecognized immunological mechanism which depends upon the development of a local refractory state in the tissue cells themselves and is independent of antibodies, phagocytosis, and the anti-infective functions of inflammation." It seems possible that further work will in fact show that the other factors of immunity, particularly antibodies, can ex plain everything once ascribed to tissue immunity, but it must b admitted that at present a number of puzzles remain to be explained

Immunity vs. Hypersensitivity

The changes which occur in an organism in response to an infectio may enable it to throw off the infection and are beneficial in the sens that resistance to subsequent infections of the same type will b greater. The mechanism of these changes, however, is evidently s general that they can be provoked by influences other than infectiou agents. Noninfectious, dead microorganisms may cause suc changes, and even apparently harmless substances may sometim cause alterations in the reactions of the tissues or the substances i the circulation. An animal may become highly reactive ("hyper sensitive") to certain harmless substances (Chapter VIII). Burn (7) has supposed that the basic mechanism of all reactions of a organism to foreign substances consists in the recognition by th tissues of the organism of the differences between "self" and "no self." You do not become hypersensitive to any of your own blo proteins, and your stomach, although it will digest tripe, refus (usually) to digest itself—so long as you are alive. The defens which an organism puts up against infection may therefore have som thing in common with the processes of digestion, just as Metchnik thought.

Not all antibodies are concerned with infection. The injection many substances not derived from disease-producing organisms w call forth the production of substances which have all the gener characteristics of antibodies, and which can be observed to react wi the injected antigen in some recognizable way. Since there is every reason to believe that these substances are of the same nature as antibodies concerned with disease, and are produced by the same mechanism, they are also called antibodies, and the substances which cause their production are called antigens.

Such "pseudo-defense" measures, in the case of usually harmless substances such as ragweed pollen, may set up a mode of reaction which is actually deleterious to the organism (Chapter VIII). We can only suppose that on the whole the benefits of possessing the immunity–sensitization mechanism outweigh the harm, or otherwise the device would not have survived during the long course of evolution.

Antibodies

Animals exposed to infection produce in the blood and tissue fluids soluble substances which tend to prevent or cure infection. These are called *antibodies* (because they are bodies acting *against* introduced substances), but the term, as we have seen, is not restricted to substances with a protective function. The substances that call forth the production by the animal of antibodies are called *antigens* (because they cause animals to *generate* the antibodies). There is a certain element of circularity in these definitions, which we shall not bother to remove, for the reader will acquire a better notion of what antibodies and antigens are by reading the following chapters than from any definition.

A substance which can act as an antigen is said to be antigenic. Some substances and mixtures which are not antigenic, or are only slightly so, are however often referred to as antigens, because they *react* with antibodies. This inconsistency in nomenclature is not as confusing as might appear at first.

9. Mechanism of Resistance to Infection

When the natural barriers of the body fail to prevent a pathogenic microorganism from gaining entrance, an infection results, and the above mechanisms begin to operate. Everybody is familiar, as Burnet (7) points out, with the typical course of a minor infection such as that following a cut in the skin, or a boil. Inflammation (42–45) sets in, leucocytes are mobilized, and other changes, such as the formation of antibodies, which are not directly observable, take place.

Topley and Wilson (63) have attempted to reconstruct what happens when bacteria gain access to the tissues of a host and succeed in gaining access to the blood stream, but are finally vanquished by the defensive mechanisms:

During the initial phase, the bacteria enter through a mucous surface and pass to a regional lymph gland. They proceed to proliferate there. Then the bacteria begin to collect in the reticuloendothelial system, for example in the liver and spleen, brought there by the normal clearing action of the blood. They continue to proliferate there and in the lymphatic glands where they entered; this phase corresponds to the incubation period. Then, if the disease is serious, the blood stream is reinvaded, bacteremia results, and bacteria are carried to other, previously uninfected tissues. This corresponds to the phase of clinical illness, and, if the host's immune mechanisms are not equal to the battle, death will soon result. If the host is going to recover, antibodies usually make their appearance during this phase. The clearing mechanisms as a consequence are speeded up, the bacteremia disappears, and only a few scattered foci of infection, for example in the spleen, liver, and lymph nodes are left. This stage may possibly persist for a long time in some cases. Finally, the remaining foci of infection may be eliminated, and the host is once more completely free from the infection.

In milder infections the stage of clinical illness may never be reached, and the infection is said to be abortive. Such infections may nevertheless produce effective immunity, especially if repeated, and protective antibodies to a variety of infections may be found in individuals who so far as is known have never had a clinically recognizable case of the disease in question.

Conclusions

In view of our ignorance of nonspecific factors in immunity, and our doubts as to the existence of tissue immunity, compared to the demonstrated importance of antibodies, acting alone or in conjunction with phagocytosis, we are pretty much forced to speak of antibodies as the main key to immunity. This has long been the prevailing view, and the result has been intensive study by large numbers of skillful investigators, so that we now know much more about antibodies than about any other aspect of the mechanism of immunity. Antibodies alone, however, may often be ineffective, as we have learned from the vital role phagocytes play. Complement (Chapter VII) may also be required.

SUMMARY

(1) Parasitism results from competition in the "struggle for existence." Mechanisms of resisting hostile organisms have developed in the course of evolution. *(2)* Immunity is used in this book to mean the degree of resistance to a disease. Some organisms have natural resistance to certain diseases. *(3)* Inflammation is a part of the protective mechanisms against infection. The sequence of events in inflammation is given on page 8. *(4)* Phagocytosis is important in the process of inflammation and of resistance in general; it is accomplished by the circulating leucocytes and histiocytes, which are part of the reticulo-endothelial system. The mechanism of phagocytosis is interpreted in physico-chemical terms and seems to depend on changes which take place first at the surface of the foreign particle. Serum, particularly that which contains antibodies, facilitates phagocytosis (opsonic action of serum). (The importance of phagocytosis is summarized on page 13). Phagocytes may, but do not always, kill bacteria which they ingest. Some virulent bacteria resist phagocytosis. *(5)* There are various kinds of immunity:

- *(I)* Innate immunity (constitutional or racial).
- *(II)* Acquired immunity.
  - *(1)* Active (make your own).
    - *(a)* Natural (the host comes in contact naturally with the disease agent).
    - *(b)* Artificial (the disease agent, or some product of it, is introduced deliberately).
  - *(2)* Passive (antibodies are furnished).
    - *(a)* Natural (congenital).
    - *(b)* Artificial.

(6) Grades of immunity range from completely susceptible to completely immune, with the intermediate grades more common. *(7)* The measurement of immunity presents problems, especially if we wish to estimate the immunity of an individual. The determination of antibody levels offers one fairly successful method. *(8)* Acquired immunity may depend on *(a)* some increase in the nonspecific factors of resistance, *(b)* alterations of the tissues in the direction of greater resistance (tissue immunity), *(c)* increase in rapidity and degree of mobilization of phagocytes, *(d)* production of antibodies. Immunity and hypersensitivity seem to be closely related. Tissue immunity, meaning an acquired resistance intrinsic in the tissues, may exist, but the evidence is doubtful. The basic mechanism which produces immunity may also be responsible for hypersensitivity. The soluble

substances appearing in the blood and tissue fluids which help counteract the infection are called antibodies. (*9*) The mechanism of resistance to infection involves all of the above factors. A sketch of the progress of a typical bacterial infection which results in victory for the host is given.

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CHAPTER II

ANTIBODIES
AND ANTIBODY SPECIFICITY

1. Introduction

Immunology doubtless impresses many a beginner as a highly theoretical subject, perhaps because of the polysyllabic terminology and sometimes slightly fantastic theories involved; but actually it is a very practical science, concerned with methods of preventing disease or influencing its course. This practical emphasis serves more or less automatically to delimit the subject. In addition to the mechanisms which we call immunological, a number of others have important effects in preventing or ameliorating disease. But since most of these mechanisms are not within our power to affect artificially, it is customary to ignore them or to consider them in connection with some other subject. Thus we shall not discuss methods of combating diseases by avoiding or eliminating the disease agent or its carriers, or the immunity to disease based on differences in habits or locality, or any mode of treatment except biological therapy.

Also, no treatment of the chemotherapeutic methods which have become so important in modern clinical medicine is attempted. After all, the student will find such subjects better discussed in other books. Furthermore, the discovery of strains of microorganisms which have become resistant to sulfa drugs (172, 180), penicillin, and streptomycin (182a) just as trypanosomes have in the past become "drug-fast," might suggest that chemotherapy is unlikely to replace immunological methods completely in the near future.

2. Chemical Constitution of Blood, Plasma, and Serum

Since the phenomena we are going to be discussing are so intimately connected with blood, a few words concerning this important fluid may be appropriate. Blood consists of a liquid portion, called plasma, in which float a number of different kinds of cells, including the oxygen-transporting red cells and the various kinds of white cells, or leucocytes, already discussed. If blood is drawn from an animal and prevented from clotting by the addition of an anticoagulant, such as sodium citrate, lithium oxalate, or heparin, the cells can be centrifuged off and the plasma obtained.

If blood is allowed to clot, certain constituents of it unite to form a fibrous mass which traps the cells, forming a firm, jelly-like clot. This clot, on standing, will shrink, and a clear yellowish fluid, called serum, separates. Serum is similar in composition to plasma, but differs somewhat, chiefly in the absence of the fibrinogen (a protein making up about 6% of all the plasma proteins), which has entered into the composition of the clot.

There remain in the serum a number of inorganic dissolved substances, various more or less simple organic compounds, some containing nitrogen, and the high molecular weight nitrogenous compounds called proteins. The great majority of these proteins remaining can be placed in one of two classes, originally based on solubility characteristics, called globulins and albumins. Since the first chemical work was done on these proteins, other differences have been discovered, such as differences in molecular size and net electrical charge per molecule. Antibodies are found to belong to the class called globulins. The ways in which they may differ from "normal" proteins will be discussed below.

3. Nature of Antibodies

Since originally antibodies were purely hypothetical substances, they were named and characterized merely according to their effects, and many workers avoided specifying what in the serum gave it antibody properties. We now know that antibodies are actual chemical substances (modified globulins), and we can give up this excessive caution and start our discussion of antibodies immediately on the basis of modern knowledge.

4. One Antibody, Many Manifestations

It could easily be imagined that the only way of detecting antibodies would be by their power of destroying or neutralizing infectious agents. This seems to be true of some antibodies, notably those against certain viruses, and there the technic of demonstration does depend on the neutralizing effect which the antibodies have.

Fortunately for us, however, antibodies in many cases react with the antigen in a way which is or can be made visible in the test tube. Thus antibodies have been observed to cause swelling of microorganisms against which they are directed, to cause lysis (solution) of microorganisms and foreign cells, to cause agglutination (sticking together) of organisms and cells, to kill microorganisms, to cause the formation of a precipitate when mixed with soluble antigen, to opsonize (see above) microorganisms so they can be phagocyted, to neutralize toxic products of bacteria (or other toxins), and to sensitize animals passively. In addition, in most of these reactions, complement will, if present, enter into combination with the antibody-antigen compound (see Chapter VII). By testing later for diminution of complement concentration, the fact that an antibody-antigen reaction has taken place can be determined, and thus the presence of antibody inferred.

Originally a separate name was given to the hypothetical cause of each different serological phenomenon, giving us names such as lysin, bacteriolysin, hemolysin, amboceptor, agglutinin, hemagglutinin, bactericidin, precipitin, opsonin, tropin, bacteriotropin, antitoxin, sensitizer, etc. Eventually it occurred to immunologists (5, 39, 194) that it might be possible for the same antibody to perform all or most of these functions, and thus the number of hypothetical entities required was drastically reduced. This theory has been called the "unitarian" view, and Zinsser (195) thus formulated it:

"By such a conception it is not implied that a complex cell like, for instance, the typhoid bacillus gives rise to one variety of antibody only. There may be formed a specific sensitizing antibody against the major chemical constituent, and other sensitizers against other antigenic substances enclosed in the same cell body or contained in the same antigenic solution. But we do mean that, were we working with a single antigen, in a pure state, one variety of antibody only would be produced. This would be present in the form of a serum constituent specifically capable of uniting with the antigen. As a result of the union, the antigen is altered in its physical and in its chemical behavior. The resultant reactions which may be observed with this sensitized antigen (agglutination, precipitation, complement fixation, bactericidal phenomena, bacteriolysis, opsonization, or sensitizing effects in the anaphylactic sense) are determined not by differences in the nature of the antibodies with which the antigen has united, but rather by the physical state of the antigen itself, the nature of the co-operative substances (alexin, leucocytes, tissue cells) and by the environmental conditions under which the observations are made."

Evidence has since been presented which indicates that a single pure antigen may produce two or more antibodies of somewhat different specificities (see page 37). Therefore the unitarian hypothesis, if we wish to retain it, reduces simply to the proposition contained in the last sentence of Zinsser's formulation, and may perhaps also have to be emended to allow for the possibility that the chemical nature of different antibodies may also affect somewhat the nature of the secondary reaction resulting from their union with antigen.

The unitarian hypothesis in this somewhat restricted sense has been supported experimentally by studies such as those of Chow and Wu (29), Doerr and Russ (43), Heidelberger and Kabat (84), and Kabat and Bezer (113). Heidelberger and Kabat found that type-specific agglutinin and precipitin were present in identical amounts in type I antipneumococcus horse serum, and a reduction in one was accompanied by a quantitatively identical reduction in the other, indicating their actual identity. Doerr and Russ found the anaphylactic sensitizing effect of sera to be quantitatively proportional to the precipitin content, in experiments which justify us in identifying precipitating and anaphylactic antibodies. Chow and Wu found that the immunologically pure precipitin from rabbit sera would protect mice from an otherwise fatal dose of type I pneumococcus, produce passive anaphylaxis, and fix complement, as well as, of course, precipitate its homologous polysaccharide.

5. Modes of Behavior

In most cases study has not revealed any differences between antibodies and globulins normally present in the blood which are great enough to enable the presence of antibodies to be recognized chemically. The presence of an unusual amount of globulin, particularly of "euglobulin" (as determined by salting out), probably indicates the presence of antibody, and similarly an increase in the amount of special globulin components found by electrophoretic or ultracentrifugal examination indicates the presence of antibodies in certain horse antiprotein sera (page 43). However, none of these methods can detect the presence of very small amounts of antibody, and in addition the methods suffer from a much more serious drawback; they are not specific, for they do not tell us *which* antibodies are present. Therefore the best methods for the recognition of antibodies depend upon the characteristic behavior which they exhibit when brought in contact with the antigen under the appropriate conditions. The test for protective power by testing with mixtures of antiserum and infective agent is in some cases the only one at our disposal, and consequently has to be used. It has the merit that it tests for the thing we are interested in, immunity, since we know that this is dependent chiefly on the properties of the blood. The test is however wasteful of experimental animals, time consuming, and even when large numbers of animals are used, still subject to some residual uncertainty (see Chapter XI). Some antibodies are directed against toxic products (toxins) produced by the infective organism, and will neutralize these substances, rendering them harmless. The presence of these antibodies can be tested for by mixing the serum with the toxin (freed from the microorganisms) and then testing the toxicity of the mixture on suitable susceptible animals. This has some of the disadvantages of the previous method, but, similarly, in some cases it is still the best method available.

In view of the technical difficulties of the direct test on animals, and because other antibodies which interest us seem to have little protective power, and because we find it instructive to study, in experimental investigations, antibodies to noninfective and nontoxic substances and cells, it is fortunate that we have in most cases other ways of recognizing the presence of antibodies.

An antibody directed towards a soluble constituent of a microorganism, or against other soluble antigens, will usually, when mixed in appropriate concentration with a solution of the antigen, produce a precipitate. Such precipitates, which contain both antibody and antigen, when made for example with toxin and antitoxin in appropriate amounts, are found to be neutral (i.e., nontoxic) and to have a definite composition. Since this procedure can be carried out in the test tube, it offers great advantages over tests requiring animals. In addition it is rapid, easily seen, and well suited for demonstration. It is natural that this *precipitin reaction* (see Fig. 4) has become one of the most extensively used of the immunological reactions.

It has also been found that antibodies directed against a microorganism or a foreign cell will often, when mixed in suitable concentration with a suspension of these cells, cause them to stick together in clumps. This is called *agglutination*, and provides a rapid and striking method of detecting antibodies (see Fig. 5).

Conglutinin is the name applied to a colloidal substance, occurring

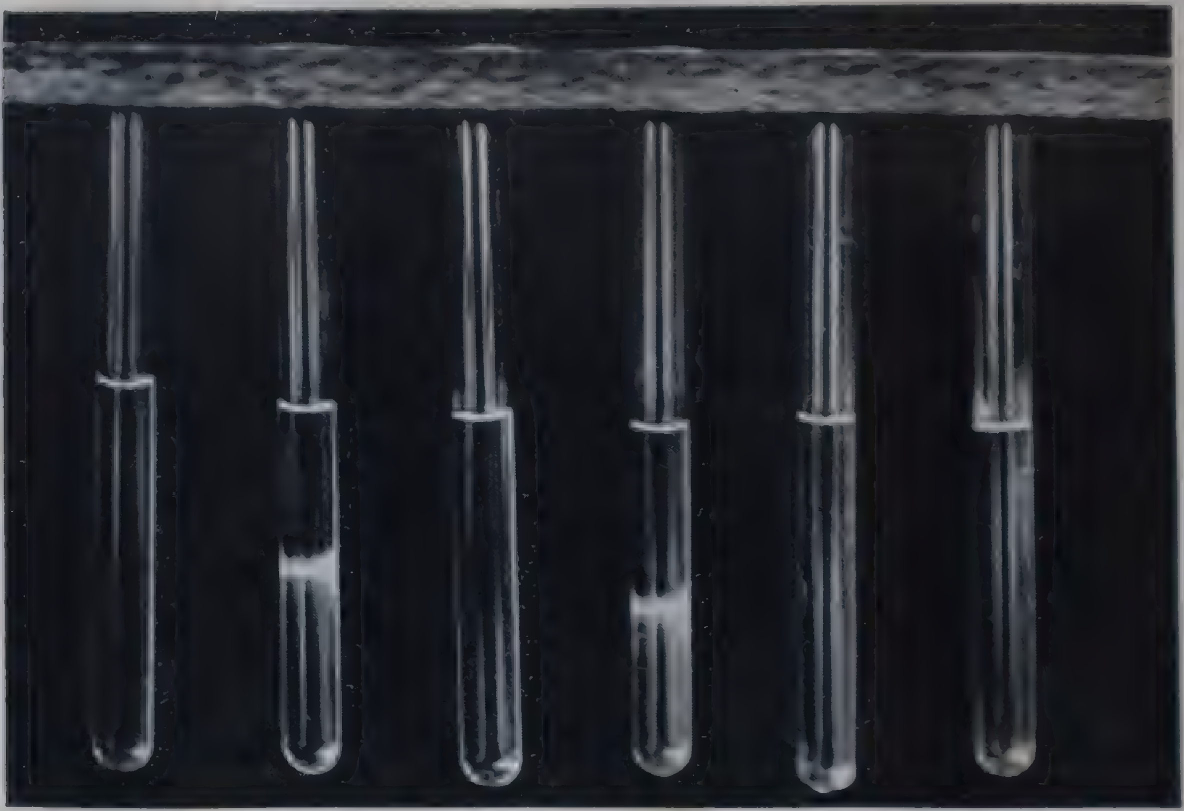

Fig. 4. Photograph of precipitin tests (168a). In tubes 2 and 4, counting from the left, a positive reaction is seen at the interface between serum and antigen. Tubes 1, 3, 5, and 6 are negative. This test was carried out by the older (Uhlenhuth) technic, which uses larger tubes, and consequently requires larger amounts of reagents than the technic recommended here.

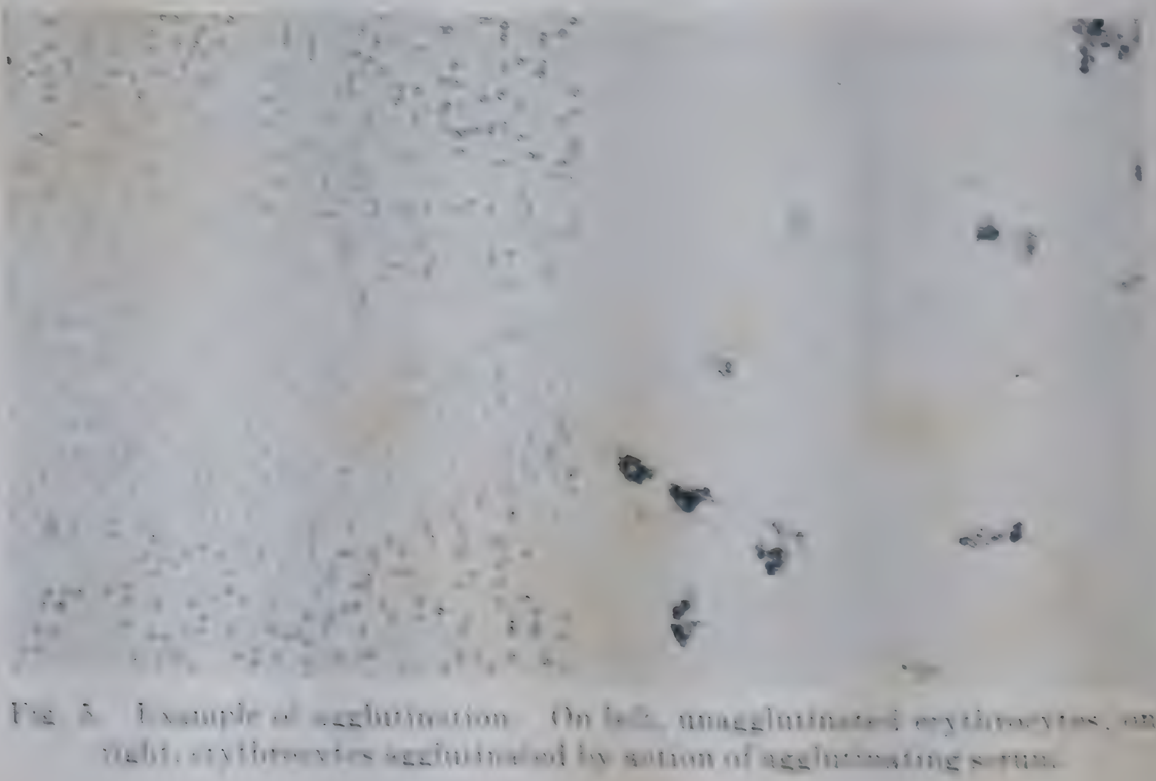

Fig. 5. Example of agglutination. On left, unagglutinated erythrocytes; on right, erythrocytes agglutinated by action of agglutinating serum.

especially in cow serum, which combines with cells after they have combined with antibody and enhances agglutination (and lysis).

In some cases the antibody is observed to kill the microorganism responsible for its production (*bactericidal* effect). Such antibodies can cause partial or complete solution of the cells against which they are directed. This is called *lysis*, and requires the cooperation of of other substances found in serum (complement).

In some cases the antibody has the power of causing the microorganism to swell visibly (German, *Quellung*), and thus provides a convenient method of recognizing its presence (see Chapter XI, Fig. 45, page 419).

We have seen that combination of antibody with microorganisms seems in most cases either essential to, or of enormous importance for, *phagocytosis*. Therefore, by treating the organisms with the serum and then exposing them to the action of leucocytes, the presence of antibody can be detected.

Some antibodies combine with their antigens without producing any visible change. If the antibody–antigen compound is capable of combining with complement, as is usually the case, the reaction causes complement to disappear from the serum (*complement fixation*). This also is not accompanied by any visible change, but may be detected indirectly by adding an antibody-antigen system which, in the presence of complement, but not without it, will produce some visible phenomenon (lysis). This is the basis of the Wassermann test, where the presence of the syphilitic "reagin" (antibody) is detected by adding an antigen with which it will react, if it is present, causing added complement to be taken up at the same time. Then sheep erythrocytes and antisheep lysin are added. If lysis of the sheep cells takes place, this shows that complement is still present in the serum and no reaction took place with the Wassermann antigen, consequently no "reagin" was present; if lysis does not take place, it indicates the presence of "reagin," and presumably of syphilitic infection. See Chapters VI and XI.

Immunized animals often become hyperreactive to the antigen ("sensitive" or "allergic"). In many cases the antibody in their circulation is capable, if injected into a normal animal, of making the latter sensitive also (passive *sensitization*). This can be detected by appropriate tests of the passively sensitized animal, and provides another indirect way of demonstrating antibodies.

6. SPECIFICITY

It is an old observation that, although recovery from a disease may give an individual a longer or shorter period of immunity to this disease, it does not protect him against other unrelated diseases. If antibodies are one of the main bases of immunity, we should expect that they would also show this selective action, and this is the case.

Antibodies which neutralize diphtheria toxin (antitoxins) will give protection against diphtheria and will aid in the cure of the disease; they are of no use in streptococcal infections or syphilis. Diphtheria antitoxin will produce a precipitate when mixed in the proper proportions with diphtheria toxin; it will not precipitate tetanus toxin. Antibodies can apparently recognize the difference between proteins from different species, and between different proteins of the same species, and each can single out its own antigen. There is a limit, however, even to the astonishing precision of serological specificity. When homologous proteins of closely related species or carbohydrates from related species are used, the antibodies for one antigen may react also with another, though usually less strongly. In some cases, particularly with carbohydrates, reactions may occur with substances of quite a different biological origin.

The specificity of serological reactions, extraordinarily sharp, but still limited, has been well defined by Landsteiner (119) as "the disproportional action of a number of similar agents on a variety of related substrata." This definition seems the best that has been proposed, although it would also include many chemical reactions, as might be expected if serological specificity were fundamentally chemical in its nature, which we now believe.

Much of what we know about the specificity of antibodies has been learned by the study of antibodies to proteins which have had coupled to them relatively simple compounds of known composition. Antibodies to simple, known, introduced groups (haptens) could often thus be obtained, and a study of their reactions was extremely informative. We shall discuss methods of altering antigens chemically in the next chapter.

Probably the most outstanding investigator in this field was Karl Landsteiner, and the second edition of his monumental book (120), which he fortunately had completed just before his death, will long be the best source of information for the student of the specificity of antibodies and antigens. In a textbook such as the present, it would be both impossible and undesirable to cover the field in the complete way Landsteiner did.

7. NONHOMOGENEITY

It will be seen in Chapter III that antibodies to conjugated antigens may be of three kinds: one directed towards the hapten, one towards the protein carrier, and occasionally, apparently, against both simultaneously. We could treat each of these classes as if it were a homogeneous substance, each molecule possessing the same specificity. It would be better, however, to inquire, is this the case? The weight of evidence indicates that in general an antiserum against a given antigen contains a diversity of antibodies not all alike (34, 68, 70, 86, 101–103, 118, 123, 124, 184–186).

In some instances these antibodies are distinct, that is, some are directed toward one part of the molecule, and some toward another. This has proved fairly easy to establish in the case of antibodies to conjugated antigens, but in the case of natural proteins, although it has been suggested (34, 102), it would seem that it has not yet been actually proved (126). It has also been established in at least one case for the part of the antibodies directed towards the introduced group, that is, antibodies specific for different parts of the haptenic compound have been detected (125) and may be present in other instances (4, 101, 134, 137). There is hardly any doubt that if a sufficiently large, complicated molecule, containing acid groups, were used as the hapten, distinct antibodies could almost regularly be obtained.

In the majority of cases the difference in the antibodies does not seem to mean that there are present two or more separate antibodies sharply directed towards different parts of the molecule, but rather that the antibody molecules present vary among themselves in the extent to which they are directed towards the whole hapten (see page 128) or towards the part which serves as the antigenic "determinant." Therefore, although we must assume a multiplicity of antibodies (124), we should think of them, not as several distinct species, but rather as a large family, with varying degrees of deviation from a mean. It is likely that some of the more weakly reactive molecules reflect the structure of the determinant very imperfectly, and that the more "avid" molecules have more faithfully reproduced the essential electronic configuration (in reverse, we must assume) of the original structure (76). The nature and size of the antigenic determinants in protein antigens is discussed in the following chapter.

We should also mention that the specificity of antibodies often becomes less as immunization is continued, as shown by the increase in strength and extent of cross reactions (120). To some extent this may be due to increased antibody concentration in the sera from later bleedings, but some observations may suggest that it is partly caused by the production of antibodies of greater combining capacity (120). Qualitative changes in combining power may also be observed between early and late antibodies, for it has been shown that in at least one case the later antibodies could combine with a related antigenic determinant with which the antibodies at first produced showed no reaction (99).

The individuality of the animal producing the antibody must also play a considerable role. It is common knowledge that not all rabbits respond in the same way to a given antigen. Bodily and Eaton (13) found wide variations in the specificity of the immune response of human beings to various strains of influenza A virus. This nonhomogeneity of antibodies is doubtless one of the obstacles to their chemical characterization, and may explain, for example, why it has been so difficult to prepare antibodies in definitely crystalline form.

To sum up what we know about the specificity of antibodies, we may say: antibodies can be sharply specific and can distinguish minute differences in chemical or spatial configuration. Differences which are slight from the chemical point of view tend to be slight serologically, and in some cases, especially with neutral antigenic determinants, antibodies fail to distinguish marked chemical differences. Antibodies clearly must contain spatial differences corresponding to the differences of the stereoisomeric substances they can differentiate. In some cases the specificity of antibodies is of a relatively low order, and they may be specific for a part of the molecule only, or even for certain radicals, such as the arsonic acid group ($-AsO_3H_2$) or ($-SO_3H$) (119). Antibodies may be directed towards more than one determinant in the molecule, and even those directed towards one determinant must vary to some extent around a main pattern. Antibodies will react with simple chemical compounds as well as with proteins and other complex antigens.

8. NORMAL ANTIBODIES

In the blood of animals which have not been immunized, and so far as we know have not had the disease in question, there may occur substances reacting with a certain infectious agent or with products derived from it. Also of frequent occurrence are substances acting on the erythrocytes of other species, or even on erythrocytes of different individuals of the same species. These substances are so much like antibodies in their behavior that they are called normal or natural antibodies.

Some of the "normal" antibodies are probably real immune antibodies, formed in response to the infectious agent which, however, failed to produce the disease, perhaps because too small a dose gained entrance, but which did produce a latent or subclinical infection, or at any rate some antibody. This has been pretty well proved in the case of "normal" antitoxin for diphtheria (48), and in certain other cases (6, 108, 156). There is also the possibility that in some cases the antibody production has been in response to organisms which did not enter by the usual portal of infection, and this has been affirmed for tetanus antitoxin by Ramon and Lemétayer (157).

It is also likely that some of these antibodies are produced in response, not to the organism with which we observe them to react, but to some related organism or to some unrelated organism which contains a common antigen. This seems to be the origin of the anti-sheep hemolysins which may appear in rabbits as a consequence of invasion by bacteria like *Pasteurella cuniculicida* which contain the Forssman antigen (Chapter IV). The presence of related antigens in food is probably also sometimes a factor.

However not all the normal antibodies can be accounted for in this way. Some, like the isoagglutinins in human blood (see Chapter V), are definitely hereditary in character and seem to develop spontaneously without the necessity of any antigenic stimulus. Landsteiner (119) suggests such a spontaneous origin for most of the normal hemagglutinins and hemolysins acting on the blood of foreign species. This is supported by the existence of certain regularities in the zoological distribution of these substances.

The normal antibodies exhibit considerable specificity; indeed, if they did not they would probably not be called antibodies. If part of them are really immune antibodies formed through undetected contact with the antigen, this is to be expected; but even those which presumably originated otherwise exhibit some degree of specificity. It might have been thought that the agglutination and lysis of bacteria and blood corpuscles by the serum of normal animals was due to one or a few substances, each capable of acting on practically any sort of cell. This is not the case, however, since absorption of the antibodies affecting one sort of cell usually leaves the activity for other sorts undiminished. An example of this is shown in Table I, based on work of Malkoff (132).

Malkoff, followed by many others, concluded that a normal serum contains as many specific agglutinins as there are sorts of cells that are agglutinated by the serum. But the number of cells and microorganisms agglutinated by some normal sera is very great, and it is scarcely possible that such a large number of antibodies is present. If so many were present, they (being globulins) would raise the globulin content of the serum to values higher than any ever observed; otherwise all or the majority of them could be present only in amounts hardly more than infinitesimal. In that case they could hardly act as strongly as we observe them to. Actually, experiments indicate that a fair amount is present (122). Landsteiner (119) also found that if agglutinin from normal serum is set free from the agglutinated cells it will agglutinate, in addition to the cells used in absorbing it, those of other species, which is contrary to what would be expected from Malkoff's idea.

TABLE I

AGGLUTINATION OF VARIOUS ANIMAL ERYTHROCYTES BY GOAT SERUM (132)

Test against erythrocytes of	(Unabsorbed)	Goat serum absorbed with erythrocytes of				
		Pigeon	Rabbit	Man	Rabbit and pigeon	Pigeon and man
Pigeon	+	0	+	+	0	0
Rabbit	+	+	0	+	0	+
Man	+	+	+	0	+	0

The symbol + indicates agglutination; 0 indicates no agglutination.

There is no doubt that the normal agglutinins, etc., of the sort Malkoff dealt with, are not as highly specific as real immune antibodies, and it is not unlikely that their reactivity with the antigen in question is really due to some accidental correspondence in structure to the combining groups which a real immune antibody to that antigen would possess.

In some plants (e.g., certain beans) there are naturally occurring agglutinins having a certain degree of specificity in their action on the red cells of different species or even of different individuals. Landsteiner (119) suggests that the normal serum agglutinins are like these in that they are specific only in so far as they act to a different degree on various cells. He considers that it would be sufficient to assume that normal serum contains a certain number of such antibodies, each reacting distinctly only with a certain proportion of all bloods. From such a mixture any given cell would remove all the agglutinins for which it had affinity, leaving some that react with the cells of other species.

9. Chemical Nature

We have already stated that antibodies are serum proteins, with physical properties indicating they belong in the "globulin" class. An antibody clearly must differ in some respect from the other globulins in the serum, since it bears the imprint of its characteristic specificity, not possessed by the other globulins, and this specificity must reside in some chemical difference in at least part of the molecule. Nevertheless in most cases no marked chemical or physical differences have been observed between antibodies and so-called "normal" globulins.

That chemical differences between different antibodies sometimes exist, however, is shown by a number of observations. It has been found that the antipneumococcus antibody in horse serum has an isoelectric point of about 4.8 (91, 177, 178), while the isoelectric point of normal horse globulin ranges around 5.7 (91). (In the rabbit, antipneumococcus antibodies seem to have an isoelectric point of about 6.6, not far from that of normal rabbit globulins (67, 91.) Green *et al.* (73) found that the water-insoluble globulin from horse antipneumococcus sera has a viscosity different from any of the normal proteins, and its titration curve differs slightly from that of the normal globulin (see also Goodner and Horsfall, 70). The antipneumococcus antibodies in horse serum usually have a much higher molecular weight than the majority of the horse serum globulins, although a small amount of globulin with this molecular weight is also found in normal horse serum and probably also in normal human serum (see page 45). Different antibodies have been found to vary in resistance to high pressures (15), heat, acid, or alkali (117, 120). For instance, agglutinins for the flagellar antigens of the typhoid bacillus are more resistant to heat than are the antibodies to the somatic O antigens. These agglutinins can largely be separated from each other by fractionation of serum with alcohol under suitable conditions (33).

In their susceptibility to the action of various denaturing and destructive agents, antibodies closely resemble proteins in general. A good summary of their reactions will be found in Marrack's book

(133). They are destroyed by heat very much as proteins are; in many instances the course is that of a monomolecular reaction, with a very high heat of activation, as in protein denaturation, which is probably what the process is. Aggregation of the protein molecules in the serum also takes place (9). Antibodies are usually destroyed by alcohol at room temperature, but in the cold, or if the alcohol concentration is brought rapidly to over 90%, this destructive action is much less marked. In fact, if the solutions are cooled and kept cold (about 0° to −10°C., alcohol can be used to precipitate and fractionate antibodies (32). (See page 53.) Treatment with protein-altering reagents, such as diazonium compounds, iodine, formaldehyde, and ketene, usually reduces or destroys the activity of antibodies.

Some antibodies have been reported to be destroyed by pepsin, less rapidly by trypsin. Parfentjev (144) found that treatment of diphtheria antitoxic serum with pepsin until 70–80% of the protein was rendered noncoagulable by heat resulted in considerable purification, giving a product a high proportion of which was specifically antitoxic (see 183). Similar treatment of horse antipneumococcus serum destroyed practically all the mouse protective power, although considerable precipitating power remained (168). It has been found (149) that digested horse antipneumococcus (types 1 and 2) antibody combines with twice as much polysaccharide per milligram of antibody nitrogen as does the normal antibody. Petermann and Pappenheimer (149) found, no matter what the molecular weight of the antibody in the starting material for this product, a sedimentation constant of 5.2 suggesting a final molecular weight of less than 100,000.

10. Molecular Weight

Molecular weights have been determined for a number of antibodies, usually by the Svedberg ultracentrifuge technic (120). The speed with which particles settle out of a suspension depends, among other factors, upon their size. The same applies to sedimentation in the artificial gravitational fields which can be produced by whirling the suspension (or solution) rapidly around an axis in the devices called centrifuges. Svedberg (175) devised an apparatus which spun the solution sufficiently fast, and at the same time guarded against disturbing effects such as the setting up of convection currents, so that the rate of sedimentation, even of submicroscopic particles such as viruses and protein molecules, could be measured. This device he called an ultracentrifuge. The "sedimentation constant" is a measure of the rate at which the particles or molecules move in a unit centrifugal field.

In order to appreciate the changes which occur on immunization, a knowledge of the centrifugal behavior of normal sera is necessary. In the ultracentrifuge normal horse serum shows two main components, with sedimentation constants (S_{20}) of about 4.5 and 7.1, respectively. These correspond to the sedimentation constants of serum albumin and serum globulin, but if the relative amounts are estimated from the areas of the curves obtained, it is found (115) that too high an albumin: globulin ratio (about 60:40) is obtained, compared with the more accurate values obtained from ultracentrifugal study of dilute sera and from Tiselius determinations (see below) (115, 175). Therefore it is important, if undiluted antisera are being studied, to compare them with undiluted normal serum. In addition to these two components, there is a small quantity of a molecular species having an S_{20} of 18 (175). Rabbit serum behaves similarly, but does not seem to contain the heavy (S_{20} = 18) component. Other mammalian sera differ in minor ways. In some species small amounts of an "X" globulin are found with a less well-defined sedimentation constant in the neighborhood of that of serum albumin but varying greatly with the density of the solvent (148).

The changes produced by immunization apparently depend on the species being immunized, the nature of the antigen being administered, and possibly on the route of injection (94). When a horse is injected with a protein antigen, such as toxin, the injections are almost invariably given subcutaneously. The proportion of globulin in the serum is observed to increase, and at least part of this increase is due to the presence of antibody having a sedimentation constant of about 7, which is apparently not different from that of normal globulin (115, 143). Bacteria such as pneumococci are generally injected intravenously into the horse, and as a rule much or most of the antibody globulin produced is heavier than normal globulin, often with a sedimentation constant of about 18 (90, 168). The rabbit, injected with either sort of antigen, apparently by either route, produces antibodies having the same molecular weight as normal serum globulins (111).

The sedimentation constant of a molecule does not enable its molecular weight to be calculated from this datum alone, unless the partial specific volume and shape (or some measurement dependent on the shape) are known. Most antibody molecules seem to have about the same partial specific volume, so if data such as the diffusion constants are available, the molecular weight can be calculated. If the molecule is known to be spherical, it is not necessary to know the diffusion constant, and the molecular weight is given approximately by the following formula:

$$M = 5150 \times S_{20}^{3/2}$$

TABLE II

MOLECULAR WEIGHTS FROM ULTRACENTRIFUGE STUDIES[a]

Protein	Sedimentation constant, S_{20}	Partial specific volume, V_1	Frictional ratio, f/f_0	Molecular weight	
				Assuming spherical shape	Corrected for shape
Bacillus phlei protein	1.8	0.748	1.22	13,000	17,000
Tubercle bacillus (human) protein	3.3	0.70	1.25	23,000	32,000
Serum albumin (human)	4.6	0.733	1.28	47,000	69,000
Diphtheria toxin	4.6	0.736	1.30	49,000	72,000
Antipneumococcus antibody (rabbit)	7.0			95,000	157,000
Serum γ globulin (human)	7.2	0.739	1.38	96,000	156,000
Phycoerythrin (*Ceramium*)	12.0	0.746	1.21	220,000	290,000
Serum globulin (*Lamphetra*)	12.0	(0.745)	1.41	220,000	360,000
Urease (Jack bean)	18.6	0.73	1.19	370,000	480,000
Thyroglobulin (pig)	19.2	0.72	1.43	370,000	630,000
Antipneumococcus antibody (horse)	19.3	0.715	1.86	360,000	910,000

[a] These figures illustrate the importance of allowing for the asymmetry of the molecule, as by using the values of the frictional ratio, f/f_0, in the calculation of molecular weights. The table also shows the wide range of molecular weights found for various proteins. For aid in compiling this table I am indebted to Dr. J. L. Oncley. The data are taken from references 113, 141a, 149a, and 175.

Table II gives the molecular weights of molecules corresponding to certain commonly observed sedimentation constants. Unfortunately, most protein molecules are not spherical (Fig. 6), but seem to be instead prolate spheroids (i.e., cigar-shaped), so the first values in the table merely give minimum values for the molecular weights. Thus the molecular weight of the horse antipneumococcus antibody, which has a sedimentation constant of $S_{20} = 18$, from the table would be 394,000. Actually it is thought to be nearly 1,000,000 (56). Since in many cases, however, all that is known at all accurately about an antibody is its sedimentation constant, Table II should often be helpful in connection with current immunological literature. It always allows a minimum estimate to be made.

By determining both the sedimentation constant and the sedimentation equilibrium (175), or by other means, the "dissymmetry constant," f/f_0 can be found. Multiplying the values in the fifth column of Table II by $(f/f_0)^{3/2}$ will give approximately the correct molecular weight.

The molecular weight of antibodies in the rabbit (and probably in man) seems to be the same as that of normal rabbit globulin, i.e., about 160,000, or in some cases somewhat more. For human antipneumococcus antibody a sedimentation constant of 7.4 and a molecular weight of 195,000 (113) have been reported. Davis et al. (38) found that Wassermann antibody (syphilitic reagin) dissociated from the antibody–antigen precipitate and showed two components with sedimentation constants of 7 and 19, respectively. Other workers have reported a high molecular weight for this antibody (120).

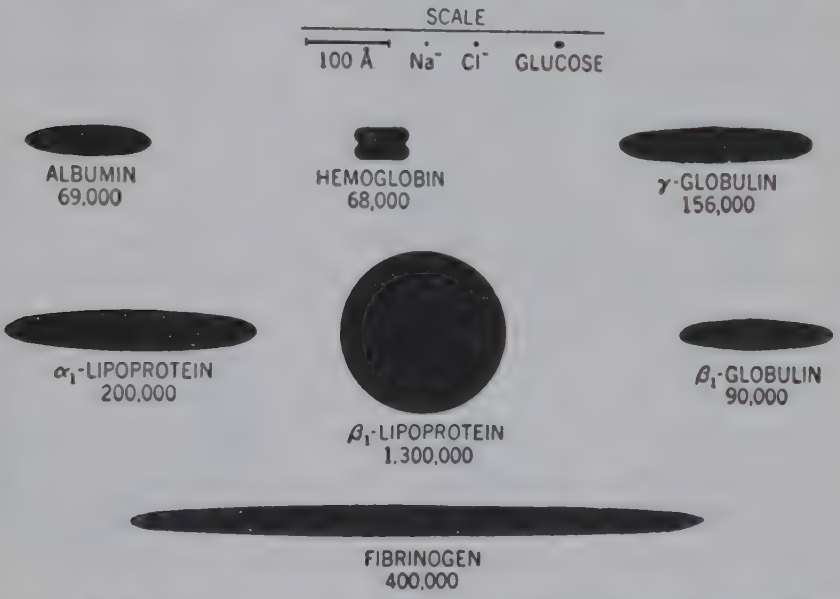
SCALE
100 Å
Na⁻
Cl⁻
GLUCOSE
ALBUMIN 69,000
HEMOGLOBIN 68,000
γ-GLOBULIN 156,000
α₁-LIPOPROTEIN 200,000
β₁-LIPOPROTEIN 1,300,000
β₁-GLOBULIN 90,000
FIBRINOGEN 400,000

Fig. 6. Relative sizes and shapes of certain protein molecules (*courtesy of Dr. J. L. Oncley*).

11. Shape of Antibody Molecules

Most antibody molecules seem to be asymmetrical (Fig. 6). Neurath (139) has calculated that in a molecule of human antipneumococcus antibody the longer and shorter axes are in the ratio of 9.2, the shorter axis being 37 Å. in length, and the longer axis 338 Å. For rabbit antibody the axis ratio is 7.5, and the lengths of the axis 37 and 274 Å. For horse antipneumococcus antibody these figures become 20.1, 47 Å. and 950 Å. For horse antitoxin (diphtheria) Petermann and Pappenheimer (149) calculate that the ratio of the major to the minor axis is 7.0.

12. Electrophoretic Mobilities

The development by Tiselius of a practical apparatus for the determination of rates of electrophoresis under controlled conditions enabled proteins to be characterized by a new method, based on their rate of migration in an electric field of known strength. This rate varies with the charge on the molecules at the *p*H in question. Use of the Tiselius apparatus has sometimes allowed the separation of proteins which were apparently homogeneous. The number of distinguishable mobilities is limited, however, and quite different particles have also been observed to migrate at the same rate.

Normal horse serum shows components with four different electrophoretic mobilities; in order of decreasing mobilities these are the albumin, and three globulin components designated as α, β, and γ globulins (177). These three globulins evidently differ in net charge per molecule at the alkaline *p*H used in the determination but are all believed to have the same sedimentation constant, $S_{20} = 7.1$; the heavy component ($S_{20} = 18$) probably migrates with the α globulin (115). Rabbit serum shows four components of approximately the same mobilities as the components of horse serum (Fig. 7). The α and β globulins of horse serum, at any rate, seem actually to be complex, each group consisting of two components designated as α_1 and α_2, and β_1 and β_2, respectively.

The electrophoretic mobilities of various antibodies have been determined by Tiselius and Kabat (178), van der Scheer *et al.* (167), and others. Their results are shown in Table III. It will be seen that the mobilities of the various components in the sera from the several animal species are approximately the same. In man, rabbit, and monkey, immune sera did not show any electrophoretic component not found in normal sera, but in the horse antiprotein sera a new component (T, or β_2), between the β and γ components, appeared after immunization. This new component seems to be all antibody, since it is absent from the same serum after absorption of the antibody.

In practice, comparison of electrophoretic patterns and of the areas under the respective peaks, rather than determination of actual electrophoretic rates, usually proves sufficiently informative.

It appears that horse antidiphtheria toxin can have the characteristics of either a β (β_2) or a γ globulin. These two kinds of antitoxin show definite differences in serological behavior. Electrophoretically pure β_2 antitoxin flocculates more slowly than the γ antibody, and has a lower L_+/L_f ratio (terms explained on pages 409, 438). The precipitate formed by the most rapidly flocculating mixture of antibody and toxin contains, when β_2 antitoxin is used, only half as much antibody per unit of toxin as when γ antitoxin is used (114, 115).

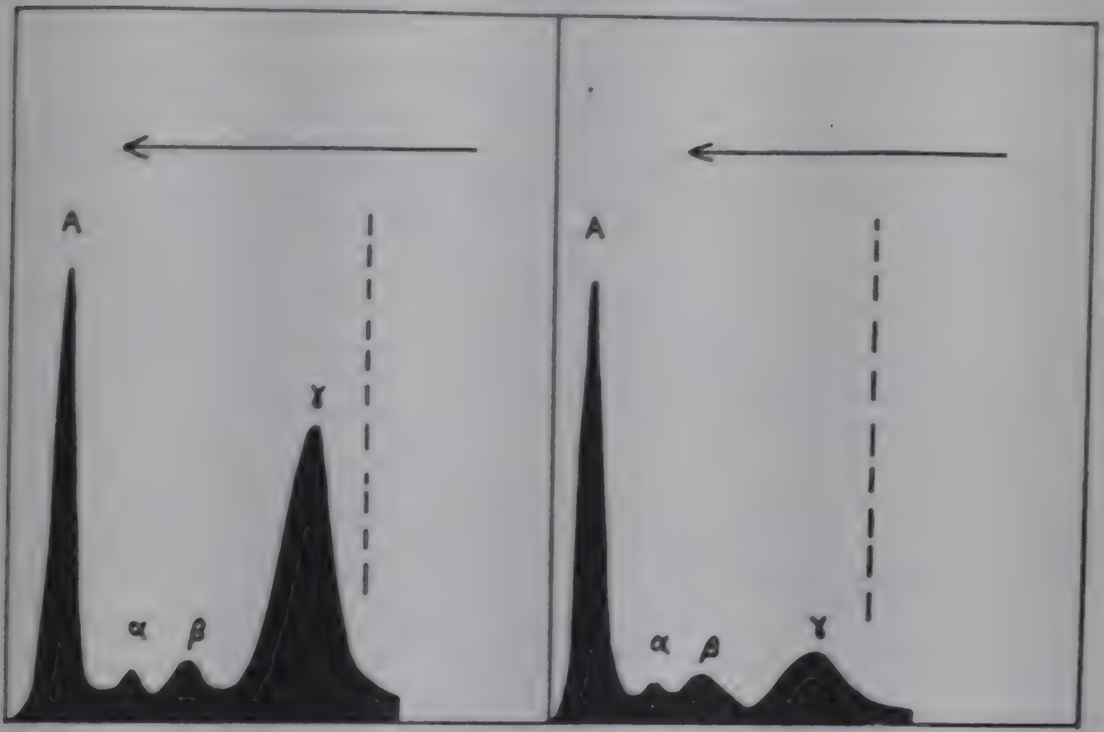

Fig. 7. Electrophoretic pattern of an immune rabbit serum before (left), and after (right) removal of antibody. Dotted lines show initial position of boundaries; arrows direction of migration. "A" signifies albumin; α, β, and γ designate α, β, and γ globulins, respectively. Areas under the various peaks may be taken as proportional to the amounts of these components present.

Rabbit antibody seems generally to have an electrophoretic mobility corresponding to that of the γ globulins. Tiselius and Kabat (178) reported that in rabbit and monkey antisera the percentage of antibody in the serum and in the γ globulin fraction can be determined by integration (i.e., determining the relative area under the curves) of the electrophoresis diagrams of unabsorbed and absorbed sera. Van der Scheer *et al.* (167) agreed that the relative prominence of the γ peak offers a fair index of the amount of antibody. Antibody solutions of high purity were obtained by Tiselius and Kabat (178) by the electrophoretic isolation of the γ globulin of rabbit antisera in which the ratio of antibody to total γ globulin was high.

Seibert and Nelson (170) found that an antibody to tuberculin protein in rabbits had about the electrophoretic rate of an α-globulin. Davis (38) found that Wassermann antibody (syphilitic reagin) dissociated from precipitates formed with the antigen had a mobility between that of the β and γ globulins.

TABLE III

ELECTROPHORETIC MOBILITIES OF THE COMPONENTS OF SERA FROM VARIOUS ANIMAL SPECIES (168, 178)

Species	Antibody for	AN/ΣN, %	$u \times 10^5$ cm^2 sec^{-1} volt^{-1}				
			Al	α	β	T	γ
Horse	Pneumococcus (types 1, 2, 5, 7, 8, 23)	20–30	5.1	3.6	2.9	—	0.9
Horse[a]	[d]	0	5.7	3.8	3.1	—	1.0
Pig[a]	Pneumococcus (type 1)	1.6	5.7	3.5	2.7	—	1.1
Rabbit[a]	Ovalbumin	36.4	6.0	3.6	2.9	—	1.1
Monkey[a]	Pneumococcus (type 3)	6.6	5.2	4.3	3.0	—	0.7
Horse[b]	Diphtheria toxin	18.9	—	4.9	—	2.6	—
Horse[c]	Diphtheria toxin	—	—	4	—	—	—

[a] Determined at pH 7.72 ± 0.02 in buffer containing 0.15 *M* NaCl and 0.02 *M* PO_4^{3-}. Temperature +0.5°C.

[b] Determined at pH 7.35, ionic strength 0.1 (143).

[c] At pH 7.3 (140).

[d] Normal horse serum (168, 178).

α, β, γ = globulin fractions; A = antibody, Al = albumin, T = (special) antibody component in horse antiprotein sera.

13. CHEMICAL ANALYSES

There have been a number of analyses for total nitrogen, amide nitrogen, mono- and diamino nitrogen, and amino acids, in antibodies and in specific precipitates containing a non-nitrogenous antigen.

The chief results are summarized in Marrack (133). On the whole, the differences between samples, and between antibody and normal globulin, do not seem to be greater than could be accounted for by the use of different methods by different workers. Antibodies and normal γ globulins have about the same chemical composition. This indicates, within the accuracy of these determinations, either that the chemical differences between antibodies and normal globulins are slight, or that they depend chiefly on arrangement of the groups within the molecules (see 145).

14. RELATION OF ELECTROPHORETIC COMPONENTS TO FRACTIONS OBTAINED BY SALTING OUT

Before the development of the Tiselius apparatus, it was common to characterize serum proteins by their solubility relations, especially in concentrated solutions of salts such as sodium and ammonium sulfate. There is no simple exact correspondence between globulin fractions so obtained and the components of different electrophoretic mobilities. However, Cohn *et al.* (31) and Svensson (176) report that there is a general parallelism between solubility and mobility, so that if relatively low salt concentrations are used, the slower moving component (γ) is precipitated completely before all of the faster components are. As a rule, however, globulin fractions thrown down by salting out consist of mixtures of components of all three mobilities. In particular the α and β globulins come down over a rather wide range of salt concentrations, suggesting that they are more inhomogeneous chemically than they are electrophoretically.

It has also been the practice of some workers to precipitate a "euglobulin" fraction from serum by dialysis (usually at physiological *p*H) against distilled water or by electrodialysis. Svensson (176) finds that euglobulin so prepared generally contains all the electrophoretic components of total globulin; he thinks that there is no clear correlation between mobility and water insolubility.

15. SPECIES DIFFERENCES

There was probably at first a tendency on the part of certain immunologists to think and speak of antibodies as if their nature would be a function solely of the antigen towards which they were directed, practically ignoring the animal which produced them. This may have been easier because it was not yet fully established that the antibodies were themselves substances. But since the normal serum proteins of different species exhibit chemical differences, it is to be expected that the antibodies will also have different properties. This has been found to be the case. Antipneumococcal antibodies seem to fall (111) into two groups as regards molecular weight. In the cow, horse, and pig, a heavy molecule of molecular weight of about 990,000 is chiefly formed; in man, rabbit, and monkey, the molecular size is that of the normal serum globulin (about 160,000). On the other hand, horse antitoxins have only the normal molecular weight of serum globulin (142, 168).

Goodner and Horsfall (69) observed a distribution of antibodies into two groups with respect to complement fixing ability and lipide composition. They found that complement (probably from the guinea pig) was fixed by antibodies from rabbit, rat, guinea pig, and sheep, whereas complement was not fixed by antibodies from horse, man, dog, mouse, cat, and goat. It will be observed that this list does not quite correspond with the classification based on molecular weight. Dingle *et al.* (41) also observed similar differences. Horsfall and Goodner (106) found that lecithin was necessary for the combining and agglutinating activity of horse antipneumococcus serum, whereas cephalin was necessary for the activity of the rabbit antipneumococcus sera.

The guinea pig is a poor producer of precipitating antibodies (35) but it has been found (41) that immunization of these animals with *Hemophilus influenzae*, though practically no demonstrable agglutinins or precipitins were produced, gave sera with a surprisingly effective bactericidal power *in vivo*, suggesting that antibody was present, but, perhaps because of its physical characteristics, not able to precipitate or agglutinate (see 14).

The solubility behavior of antibodies is generally the same as that of the class of serum proteins to which they belong; but here too species differences are found. Rabbit antibodies to proteins seem generally to appear first in the fraction of the globulins precipitated in 13.5% sodium sulfate solution ("euglobulin"), then as immunization progresses they are found also in the fraction precipitated between 13.5 and 17.4% ("pseudoglobulin I"), and only in powerful sera, as a rule, are they found in the fraction precipitated between 17.4 and 21.5% sodium sulfate concentration ("pseudoglobulin II") (17). During immunization these globulin fractions in the rabbit tend to increase in roughly this order. In the horse, antibodies to toxins and other subcutaneously injected proteins tend to fall chiefly in the pseudoglobulin fraction, and the results of Reymann (158) suggest that it is this fraction which increases most on immunization.

Most horse antiprotein antibodies, such as antitoxin, differ from horse anticarbohydrate antibodies or rabbit antibodies, in general, in the way in which they react with antigen. This difference is brought out by studies on the velocity of flocculation (14) or on the amount of precipitate formed by the addition of various amounts of antigen to a constant amount of serum (94). In either case the difference is found to consist in the fact that an excess of the horse antiprotein antibody inhibits, that is, it slows down the rate of flocculation, and if present in sufficient amount, prevents the formation of a precipitate altogether (14, 21). Boyd (14) has suggested that this difference depends at least partly upon the solubility differences just referred to; this suggestion will be discussed in Chapter VI. It was also found that rabbit antibodies, if a large number of sera were examined, showed similar but less marked differences among themselves, suggesting that the various antibodies might differ in solubility.

Heidelberger *et al.* (94), by injecting an alum-precipitated protein intravenously into the horse, have succeeded in obtaining an antibody which shows a precipitating behavior like that of horse anticarbohydrate sera or rabbit sera. Injection of another alum-precipitated protein into a horse subcutaneously produced antibodies of the type referred to above as the horse antiprotein type. This suggests that the route of injection may make a difference in the type of antibody produced by the horse. As we have noted above, horse antibacterial antibodies are as a rule produced by intravenous injection, while horse antitoxins and the two other horse antiprotein antibodies reported up to the time of the paper of Heidelberger *et al.* have been produced by subcutaneous injection.

Antitoxin in horse serum is usually found to some extent in all three globulin fractions as they are separated by salting out, and variations in its distribution among the fractions are found, even in the sera of the same animal examined by the same method at different times (66, 127). The findings depend partly on the method of protein precipitation used. In the goat it seems that diphtheria antitoxin has chiefly the properties of a euglobulin (8). Felton (57, 59, 60) found the antipneumococcal antibodies in horse serum to be of euglobulin character, and his widely used method of concentrating these antibodies was based on this fact.

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It is a noteworthy fact that immune antibodies are never found to be albumins, no matter what their species origin.

Since antibodies are serum proteins, it is to be expected that if they are injected into animals, they will be capable of acting as antigens and will have the characteristic specificity of the proteins they so closely resemble. An obstacle to trying this experiment directly is the difficulty of getting antibody free from other serum proteins. Since, however, washed precipitates of antibody and antigen probably contain practically 100% pure antibody, they should be suitable, and in fact injection of them has produced antisera verifying the above suggestion. Conversely, it has been found that antibodies are precipitated specifically by antisera to the serum globulins of the proper species (54, 105, 122, 171). According to Wright (191), the antigenic nature of horse antibody is essentially the same as that of horse normal γ globulin.

The species differences between rabbit and horse antibody acquired a practical aspect as the result of studies by Goodner and Horsfall (70), and rabbit antipneumococcus serum has practically replaced horse serum for clinical use. The most important of the differences between these two kinds of antibodies may be summarized (70): (*a*) Unconcentrated rabbit antiserum has a much higher protective potency for mice than has the horse serum. This is thought to be connected with the above mentioned fact that the rabbit molecule is smaller, so that in a given weight of rabbit antibody there are more molecules, enabling it to bind more of the pneumococcal polysaccharide than can an equal weight of horse antibody. (*b*) Rabbit antiserum does not show the prezone (prozone) phenomenon (failure to precipitate, etc., when antibody is in excess), which is a great drawback with horse serum, where use of amounts greater than the optimum may give no mouse protection at all. (*c*) According to Goodner *et al.* (71) the mouse protective power of the horse serum is inhibited by cholesterol and cephalin, whereas that of the rabbit is not. (*d*) The increase in antibodies during immunization is much more rapid in rabbits than in horses.

16. METHODS OF PURIFICATION

Methods of purifying antibodies fall into two main classes: (*a*) methods depending on the physical and chemical properties of antibodies; and (*b*) methods depending on the immunological properties.

(*a*) In most cases antibodies have been found too similar in properties to the other serum proteins to allow a complete separation. Some antibodies however have distinctive properties not shared by the majority of serum proteins, and in such cases remarkable purification has been effected by physical and chemical means. The methods of Felton (58, 60) for the purification of antipneumococcus antibody furnish a good example. Felton's first method depends on the fact that the antibodies are not precipitated at moderate salt concentration (0.025 *M*) at *p*H of about 5.2, but are precipitated at lower (0.005 *M*) salt concentrations at *p*H of about 6.7. The second method employs alcohol in the cold. The antibody is precipitated with some inactive protein, part of which can be removed by later precipitations at *p*H 5 in 0.025 *M* salt solutions. This, combined with further treatment, has enabled some very pure preparations to be made.

Since the serum albumin does not contain antibodies, some purification of antibodies can always be effected by removing the albumins, by precipitating the globulins with the proper amount of some salt (half-saturation with ammonium sulfate, or addition of sodium sulfate to 21.5%), and by filtering off the supernatant liquid. The precipitated globulins can be redissolved, and the salt can be removed by dialysis.

One of the globulin fractions is generally richer in antibodies than the others, so that removal of one or more of the other fractions by precipitation with a salt will give a purer preparation. Such purification may be well worth while for clinical purposes. One important reason for this is the lowered incidence of undesirable reactions following administration of such preparations.

Cohn *et al.* developed Felton's methods of fractionating plasma or serum by the use of alcohol in the cold (0 to -10°C.). The temperature, *p*H, protein and alcohol concentrations, and the ionic strength (dependent upon the salt concentration), could all be varied. Plasma was separated into four main fractions: I (containing most of the fibrinogen), II + III (containing most of the γ globulin), IV (mostly globulins), and V (largely albumin). Antibodies present in the plasma were found in fraction II + III (see Table IV). By subfractionation, relatively pure γ globulin could be obtained. This found considerable use during World War II. Some of its applications will be discussed in later chapters.

Methods depending on differences in the solubility of antibodies are the only ones which have thus far been successfully applied on a large scale. A preliminary treatment with enzymes (page 56) is sometimes also used. Some horse antibodies (see above) have been found to sediment in the ultracentrifuge at a different rate from the other proteins of the serum. Such methods can result in purification, but the amounts of serum which can thus be handled have been so far too small to have any practical importance.

TABLE IV
ANTIBODIES IN GLOBULIN FRACTION II + III DERIVED FROM HUMAN PLASMA (32)

Antibody	Type of antibody	Investigator	Concentration compared with plasma
Antidiphtheria	Antitoxin (neutralizing)	Edsall	10
Antidysentery	Agglutinins	Mudd	2–10
Antiherpes simplex	Neutralizing	Stokes	[a]
Anti-influenza (human PR 8)	Hirst inhibition	Hirst	4–8
Anti-influenza (human PR 8)	Hirst inhibition	Eaton	4
Anti-influenza (human PR 8)	Hirst inhibition	Enders	10–15
Anti-influenza (human PR 8)	Complement fixation	Enders	10–15
Anti-influenza (human PR 8)	Neutralizing	Stokes	10
Anti-influenza (human PR 8)	Neutralizing	Enders	9
Anti-influenza (swine)	Neutralizing	Stokes	10
Anti-influenza (swine)	Neutralizing	Shaffer	4
Antilymphocytic choriomeningitis	Neutralizing	Stokes	[a]
Antimeasles	Protective (human)	Stokes	
Antimumps	Complement fixation	Enders	2–10
Antiparapertussis	Agglutinins	Mudd	64
Antipertussis	Agglutinins	Mudd	4–10
Antipertussis	Agglutination	Enders	10
Antiperfringens	Mouse protection	Bradford	4–10
Antipoliomyelitis	Protective	Hall	[a]
Antipoliomyelitis	Neutralizing	Kramer	10
Antipoliomyelitis	Neutralizing	Stokes	16
Antipoliomyelitis	Rat and mouse protection	Kramer	10
Antipoliomyelitis	Rat and mouse protection	Stokes	10
Antiscarlatina	Neutralizing	Bradford	[a]
Antiscarlatina	Neutralizing	Wadsworth	5–10
Antistreptococcus	Antitoxin	Wadsworth	4–10
Antityphoid	H agglutinin	Enders	8–10
Antityphoid	O agglutinin	Enders	8–10
Antivaccinia	Neutralizing	Janeway	[a]
Isoagglutinins	Agglutinins	Boyd	8–10

[a] Activity present but no quantitative data.

At one time adsorption was a very popular method of purifying biological compounds. Some purification of antibody was effected in this way, but the results do not equal those of modern methods and the yield is extremely small. Some workers claimed to obtain antibodies free of protein by this method, but it is likely that they were misled by the fact that antibody reactions are more sensitive (occur in higher dilutions) than chemical tests for proteins; others have failed to confirm their work. A great disadvantage of all adsorption methods as applied to attempts to purify antibodies, or in fact any proteins, is that in the dilute solutions obtained denaturation is much more rapid than in concentrated solutions.

(*b*) The method by which antibody preparations of the highest purity to date have been obtained depends on their specific immunological characteristics. If the precipitate or "agglutinate" resulting from the reaction of an antiserum and its antigen is washed several times with adequate amounts of saline, the remaining compound consists almost entirely of antibody and antigen (plus small amounts of lipoid, complement, etc.). When it is possible to cause the reaction by which the compound was formed to reverse partially, free antibody may be obtained from such preparations.

A number of methods have been employed to set free some of the antibody. Dilute acid or alkali has been found effective in some cases; sodium chloride and glycine have been used, and in the case of agglutinated erythrocytes, warming and centrifuging while warm leaves free agglutinin in the supernatant. Another method, sometimes applicable, is to destroy the antigen in such compounds without damaging the antibody too much. Ramon (155) and Locke, Main, and Hirsch (129) thus recovered antitoxin from toxin-antitoxin precipitates; Sumner and Kirk (116, 174) obtained antibody from urease–antiurease precipitates by making the urease insoluble by treatment with acid.

Some of the best results have been obtained with antipneumococcus antibody. Felton (61) dissolved the precipitates made with antibody and the specific polysaccharide in calcium or strontium hydroxide. The polysaccharide was then precipitated by addition of calcium or strontium chloride and phosphate with adjustment of the *p*H; the antibody remained in solution. It could be further purified by precipitation through dialysis and resolution in salt solution.

Heidelberger, Kendall, and Teorell (89) obtained purified antibody from precipitates made with specific polysaccharide by treatment with salt concentrations ranging from 0.1 to 1.79 *M*. Chow, Lee, and Wu (28) obtained it by dissolving the precipitate in dilute alkali and then adding acid to bring the *p*H to about 9.5 when a precipitate appeared. This mixture was allowed to stand in the cold over night, then sufficient acid and sodium chloride were added to make it isotonic and bring it to *p*H 7.6. Centrifugation gave a solution containing 40–60% of the antibody. This antibody was 80–90% precipitable by the specific polysaccharide, and the authors call it "immunologically" pure.

These dissociation methods have not met with such success when applied to precipitates of antibody with a protein antigen, such as toxin, so other methods have been found necessary. The digestion method of Parfentjev, mentioned above, has resulted in some purification (accompanied however by some alteration) of diphtheria antitoxin. The procedure (168) is briefly described in Chapter XI.

Pope (151) has studied the effect of varying conditions, *p*H, time of digestion, etc., on the isolation of antibody by digestion with proteolytic enzymes and finds that antibodies are readily split by short digestion into an inactive portion, which is easily denatured by heat, and an active portion which is more resistant. The practical value of such methods depends largely on the elimination of nonspecific, antigenic proteins which they bring about. The active portions remaining seem to be nonantigenic, or at least poor antigens, so that the danger of sensitizing a patient to horse serum, and possibly of causing serum sickness or anaphylactic shock, is diminished. Coghill and colleagues (30) have used takadiastase, a preparation containing a mixture of (mostly carbohydrate-splitting) enzymes, to reduce the antigenic effect of horse sera.

It has been found by electrophoretic study (168) that a pepsin treatment first results in the disappearance of the albumin and a replacement of the "T" component by a new "γ" component. Prolonged digestion leaves this "γ" unaffected, but reduces the amount of other, electrophoretically inhomogeneous material. According to Petermann and Pappenheimer (149) the antitoxin molecule itself is split by pepsin in a plane perpendicular to the long axis, and all the antitoxic activity remains with one fragment only.

Northrop (140) reported the preparation of diphtheria antitoxin in a form which satisfies the criteria for a pure protein, by digesting away the toxin of a toxin–antitoxin precipitate with trypsin. The resulting antitoxin preparation has been obtained in several instances as crystals. The purified antitoxin was completely precipitable by diphtheria toxin. The homogeneity and freedom from denaturation of this preparation were probably due to the use of concentrated solutions during as much of the process as possible. Some modification of the antitoxin by the enzyme had probably taken place, however. Rothen (162) found that antitoxin purified by means of enzymes had a sedimentation constant of 5.5, while that which had not been so treated had a sedimentation constant of 6.9. This probably indicates that the enzyme-treated material has a somewhat lower molecular weight.

17. FORMATION OF ANTIBODIES

Place of Formation

The determination of the place of formation of the antibodies has proved to be a very difficult task. It is reasonable to assume that the antibodies are made in the same part of the body as the other serum proteins, but there does not seem to be absolute agreement as to the place where the latter originate. Madden and Whipple, who have studied this question extensively, have reviewed our knowledge of the subject (130). It seems to be their conviction that the liver is of primary importance in the production of the plasma proteins.

There is a large amount of evidence (see 179), not always too consistent, which indicates on the whole that the site of formation of antibodies is the reticulo-endothelial cell system. This has a certain basic plausibility, because of the widespread distribution of these cells, their defense role in taking up particulate matter from the circulation, and the important part they play in the phagocytic mechanism of immunity. [Also there is some indication that antibodies can be manufactured at least to some extent locally in almost any part of the body (63).] Attempts to test this idea have followed four lines of investigation: study of antibody formation after extirpation of certain organs, study of antibody formation after injury to the reticulo-endothelial system, study of antibody content of the various organs in the animal under immunization, and attempts to produce antibodies in tissue cultures.

The process of extirpating whole organs is perhaps rather radical for a satisfactory method of attacking this question, risking as it does derangement of the animal's entire metabolism. It produces many changes which are not fully understood, and the results of the method are on the whole contradictory, but there is some evidence that the organs rich in reticulo-endothelial cells have especial importance. Also possibly the observation by several workers that leucemic patients seem to possess an inferior antibody-producing power (107) may indicate an effect of injury to the reticulo-endothelial tissue. Injury to the hematopoietic centers by X-rays, benzene, etc., has been observed to decrease antibody production.

Attempts to "blockade" the reticulo-endothelial system by injection of particulate matter carrying a negative charge have figured prominently, but it can be seen at once that it is no easy matter to be sure the cells are really blocked off sufficiently to interfere with their protein-forming function. The literature has been critically reviewed by Gay (64), Jaffe (109), and Jungeblut (110).

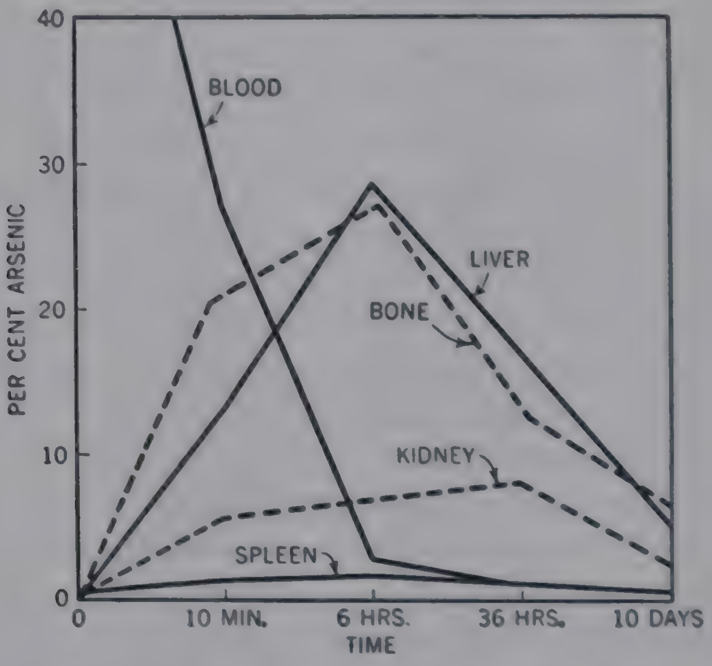

Fig. 8. Level of arsenic content of various organs following injection of arsenic-containing antigen, after various times (77).

The method of studying the antibody concentration in organs of an animal being immunized has not been used much, and some of the experiments which have been made have been started at too late a stage of immunization. There are some observations that indicate that antibody may be formed in the spleen as well as the liver (27, 181). The expedient of testing for the *antigen* in various organs at various stages has been applied on the theory that where the antigen is, there antibody is probably being produced. Haurowitz and Breinl (77) injected rabbits with an antigen prepared from arsanilic acid and determined the arsenic content of the organs after various intervals. Figure 8 shows their results. It will be seen that most antigen remained in the liver and bone marrow, i.e., in the reticulo-endothelial system. Rous and Beard (163) ingeniously demonstrated the persistence of foreign materials in the Kupffer cells of the liver (and incidentally that these cells may live for months) by using iron-containing compounds, so that the cells containing them could be separated magnetically. Sabin (164) injected a dark red dye (protein—R salt azobenzidine–azo-egg albumin) which could be identified within the cells by its color. After intravenous injections it was found in the Kupffer cells of the liver, in the macrophages of the splenic pulp, and to a minor extent in the microphages (the so-called adventitial cells) along the border of the sinusoids in the bone marrow. After intra-abdominal injection the antigen was found in the macrophages of the milk spots of the omentum and the corresponding cells of the peritoneal walls, also in the endothelial lining of the lymphatic sinuses of the retrosternal nodes and in the free macrophages of sinuses and follicles of these nodes. After intracutaneous and subcutaneous injection the antigen was found in the local macrophages and in the regional lymph nodes. The appearance of antibodies in the serum agreed with the time when the dye–protein had ceased to be visible within the cells, and a partial shedding of the surface films was occurring.

Carrel and Ingebrigtsen (26) and other workers have reported antibody formation by tissue *in vitro*. They added to tissue cultures of the bone marrow and lymph glands of guinea pigs small amounts of goat erythrocytes and obtained hemolytic substances which seemed to be specifically absorbable; they however were active without the addition of complement (see page 285). Others worked with spleen and bone marrow fragments removed after injection of the animal (see 179). Other workers have not been able to obtain such indications of antibody formation (165).

Landsteiner and Parker (121) observed the production in cultures of chicken connective tissue (fibroblasts) of proteins identical with, or closely related to, chicken serum proteins. The cultures were made in rabbit plasma and rabbit embryo juice. This seems to show that connective tissue cells can also produce such proteins.

Considerable evidence (44–46, 51, 52, 75, 114) has accumulated that lymphocytes also form antibodies. Ehrich and Harris (52) point out that we do not know the exact role of granulocytes and the macrophages, aside from that of dissolving particulate antigens (the only function of these cells which has actually been observed), and aside from their retention of antigenic material which otherwise would be excreted or destroyed elsewhere in the body. The importance of the role of the reticulo-endothelial system may or may not be diminished by these new findings (52a, 112).

18. RATE OF PRODUCTION

Antibody does not appear at once in the circulation following exposure of the animal to an antigen, nor does the animal become at once immune. Instead there is a latent period, during which time the immune mechanism is probably elaborating the antibodies which will later appear. The time required for the production of immunity may depend on the amount of antigen administered (135). Ehrlich (53) found that the immunity of mice to ricin, induced in this case by feeding, set in suddenly on the sixth day. In general, after a single injection of an antigen, there is a sudden appearance of antibody in the blood at the end of a period of several days (3). The concentration then increases, reaching a maximum in about a week (3, 22, 88). After this time, if no further injections are given, the concentration begins to fall off, rapidly at first, more gradually later, although weeks or years may elapse before the antibody content of the circulation falls to zero.

If another injection is given before this happens, the antibody present is temporarily almost or completely neutralized, but quickly begins to increase again as the introduced antigen is eliminated and new antibody is manufactured, usually to reach a higher level than before (Fig. 9). This may be repeated a number of times, and the antibody level and the rapidity with which it is restored increase, until a limit is reached. The time which must elapse before subsequent injections do not result in a final higher antibody level varies from a matter of weeks (17) to months, depending on the species. In no case can the antibody level be maintained at very high levels without periodic injections. The magnitude of this level depends partly upon the individual animal. With rabbits which have been injected repeatedly, it is likely that the highest concentration of antibody is present in the blood about five to seven days after the last injection. If injections are continued too long, the resulting antibody level of the blood may begin to decline. This may require only weeks for rabbits, or years with horses.

The absolute rate at which the organism can turn out antibody molecules is still unknown, but in view of Bjørneboe's observation

(12) of immune rabbits in which all the globulin of the blood consisted of antibody, it seems that the rate is at least as great as that at which the body can replace the globulins as they disappear at the normal rate from the blood. It has been observed (131) that a dog weighing 11 kg. can easily produce 13 g. of plasma proteins per week, or about 170 mg. per kg. of body weight per day. In diseases where antibody is constantly being taken up by a rapidly invading infectious agent, it may be that the rate of antibody production is even greater.

Evidence has been presented which indicates clearly (96, 169) that the longer persistence of antibody in the circulation of actively as opposed to passively immunized animals is due to the continued production of antibody. Passively immunized animals (merely fortified by the injection of immune serum) may lose half of the introduced antibody, even when it is from an animal of the same species, in about 72 hours (82). Cannon (25) has called attention to the probable importance of an adequate supply of protein in the diet for adequate antibody production.

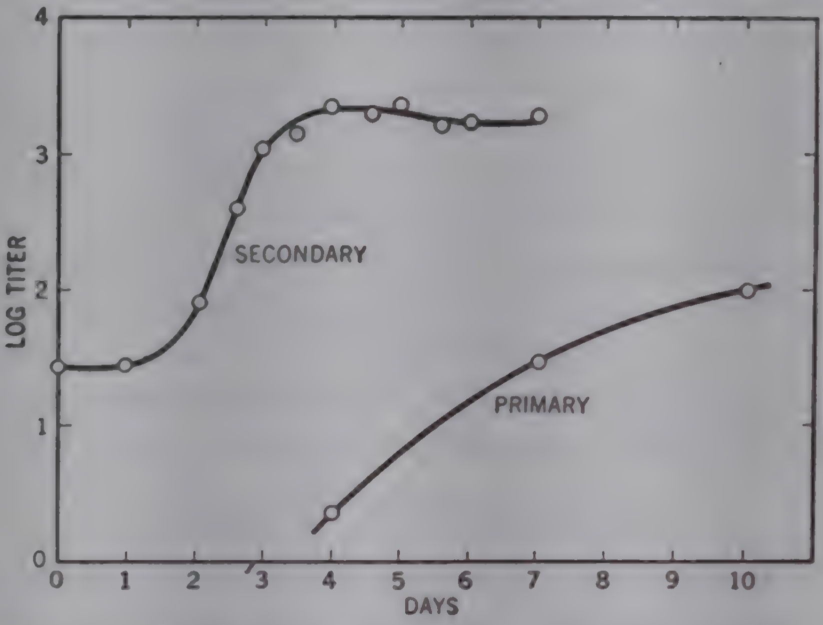

Fig. 9. Curves showing primary and secondary antibody responses (after Burnet).

19. The Anamnestic Reaction

When a specific antibody has once been produced by an animal as a result of contact of the tissues with a foreign protein, the amount of circulating antibody gradually decreases with the passing of time after contact ceases until, at length, none at all can be detected. If, now, the antigen enters the tissues again, the specific antibody will appear in the circulation and will reach a given level in a much shorter time than was required following the first contact (159). It was recognized long ago by von Pirquet (150) that when anaphylactic hypersensitivity has once been established it will gradually wane if there is no farther contact with the antigen, but reintroduction of the antigen causes the hypersensitive state to appear again in a decidedly shorter time than was required at first. Bacterial hypersensitiveness is also rapidly returned to a high level by fresh contact of the tissues with the specific bacteria (7, 188). This is called the anamnestic ("recollection") reaction (24). According to Dougherty *et al.* (46) the anamnestic response is dependent upon the release of antibody from lymphocytes as a result of pituitary–adrenal cortical stimulation.

The term is also applied to renewed production of antibodies following contact with related antigens. A number of workers have also reported renewed production of antibody following injection of quite unrelated antigens (36, 47, 180) and even nonantigenic substances (141, 182). Other workers have failed to observe this effect (161, 173) and Topley and Wilson (179) point out that Tsukahara's (180) protocols indicate that the anamnestic response was usually well marked only when the second antigen was related to the first.

20. MECHANISM OF FORMATION

The "side-chain" theory of Ehrlich supposed that antibodies were specific chemical combining groups or receptors of the cells, always present on the cells, but produced in increased numbers and cast off into the circulation following an antigenic stimulus. It now has hardly more than historical interest. It carried with it the necessity of supposing an enormous number of different preformed receptors, which alone is sufficient objection. Another early hypothesis, revived some years ago, that the injected antigen, or part of it, was in some way incorporated in the resulting antibody, has been disproved (10, 42, 80, 88, 98, 99, 100, 190).

Some have supposed that the antigen is digested preparatory to the process of antibody formation. This is improbable, since digestion would tend to destroy the characteristic structures in which specificity resides. Also antibodies can be obtained to chemical groups which the animal body can not digest. A further objection comes from Landsteiner's (119) tests on antibodies specific for fatty acids (see page 106). He found that the cross reactions between these antisera were greatest with compounds containing one less, or one more —CH_2—, and not with compounds containing two more, or two less —CH_2— groups, as would have been expected if digestive splitting off of part of the chain had been responsible for the cross reactions, for it is known that the animal body oxidizes the aliphatic chain with the loss of two carbons at a time (120).

The first theory of antibody formation proposed which still seems at all probable today is that put forward independently by Breinl and Haurowitz (20), Alexander (2), and Mudd (138) (see 98). Its chief distinguishing feature is that it assumes that antibodies are *new* globulins, synthesized (presumably in the reticulo-endothelial system) under the directing influence of a molecule or particle of antigen which is there.

It is supposed that as a new molecule of globulin is formed in the cell, the arrangement of the chemical groups on part of its surface is partly determined by the fact that a molecule, or part of a molecule, of antigen is adjacent to it. It could be imagined that the presence of a strong polar group, for example, on the surface of the antigenic particle might prevent the establishment of a polar group of like charge immediately next to it in the antibody which is being constructed, and might lead to the laying down of a group of opposite charge. In this way a configuration might result on the surface of the antibody which reflected, "in reverse," certain distinctive characteristics of part of the surface of the antigen molecule. It is necessary to suppose further either that the molecule of antibody, when completed, is cast out of the cell, and a new molecule is then formed under the influence of the persisting molecule of antigen, or that the molecule of antibody, once formed, can bring about the formation of other molecules like it; this is perhaps less likely, although it may have some analogies, as in enzyme formation for example. The chief objection to this theory of antibody formation would seem to be the fact that there is hardly any conceivable way to prove it. However, it must be admitted that the available evidence (see 77, 163, 164) accords well with it.

More recently Pauling (145) proposed a somewhat modified form of this theory. The chief differences are: *(1)* He does not suppose that the order of amino acids in the peptide chain making up the globulin molecule is affected by the presence of the antigen, but only the way in which this chain, or part of it, folds up to give the globular or elliptical molecule finally thrown off by the cell. His argument is not too easy to present without recourse to certain thermodynamic notions, but may be expressed by saying that he supposes that the two ends of the peptide chain may fold up equally readily in practically an infinite number of different ways, giving a corresponding number of characteristic patterns. He supposes that at some stage these two ends are unfolded, and fold up again under the influence of a molecule of antigen, which thus causes them to assume a configuration giving the molecule its antibody specificity. He supposes that the central part of the chain has only one stable configuration and consequently is not altered by contact with the antigen. This part is considered to bear the characteristic species specificity of the molecule. (*2*) Pauling (why is not clear) assumes the two ends of the globulin chain are both always influenced by the antigen molecule, but are never influenced simultaneously by two different molecules. We may further suppose that the extent to which the manner of folding of the ends of the chain is altered by the presence of the antigen may vary with different globulin and antigen molecules.

It is not too easy to see why the assumption that *both* ends of the globulin chain must be altered under the influence of the antigen should be an essential feature of this hypothesis. It would presumably be possible for antibodies to be formed in the manner outlined by Pauling but resulting in a molecule's having only one end folded up in a configuration enabling it to react with the antigen. Certain workers (e.g., Wiener, 187) believe that certain kinds of antibody, such as inhibiting, "incomplete," or "blocking" antibodies (see Chapter V), are univalent. Pauling, however (personal communication), states that he considers the divalence of antibody an integral part of his theory; his reasons for doing so are not clear to the present author. Haurowitz and Schwerin (79) have reported the results of a study which did not confirm the divalence of antibody, as required by Pauling's theory. This hypothesis will evidently be as hard to *prove* as the preceding ones.

Burnet (23) does not think there is any adequate reason for believing, as the authors mentioned above seem to believe, that simple juxtaposition of the growing antibody molecule to the antigen would result in a complementary pattern. Instead, he thinks it more likely that certain proteinases (or other enzymes also?) in the antibody-forming cells are lastingly modified as they are engaged in destroying the antigenic particles. Then these "trained" enzymes synthesize the antibodies. This hypothesis has the merit of a certain plausibility; data to prove it are still lacking. It hardly seems too much to say that at the present time the exact mechanism by which antibodies are formed is anybody's guess.

Pauling and Campbell (146) have claimed to be able to confer antibody-like properties on globulins and other proteins by slowly reversing (*in vitro*) the denaturation of denatured protein while it is mixed with an antigen or hapten (defined on page 128). They supposed that during denaturation the peptide chain unfolds, and when denaturation is reversed it refolds in such a way as to assume a configuration complementary to that of the antigen. They therefore interpreted their results as confirmation of the theory of antibody formation propounded by Pauling (see above). This work received considerable publicity, although the claim to have made antibodies *in vitro* is not new (50, 120, 160). A dramatized version of it was broadcast on the radio. Nevertheless, it does not seem at the present time that any outstanding immunologist believes that real antibody has ever been made artificially (112).

21. NORMAL GLOBULIN

Since antibodies are globulins, we should expect the serum globulins to increase during immunization, and this is almost always the case, particularly with the γ globulin (see references in 17). Van den Ende (55) found that the amount of γ globulin in normal rabbit serum was too small to incite the formation of any antibody for this fraction when whole rabbit serum was injected into guinea pigs, whereas injection of immune serum readily produced antibodies for the γ globulins. These antibodies reacted also with the γ globulins of normal serum.

Bjørneboe (11, 12) found that the agglutinin produced in rabbit serum during immunization with pneumococci was quantitatively identical with the increase in serum protein. In other cases, the increase in serum globulins has been found to be somewhat greater than the antibody produced. Liu, Chow, and Lee (128) wrote: "At most 2/3, usually 1/2 of the increase in globulin in a rabbit's serum, on immunization with pneumococci, is accounted for by the antibody." Boyd (17) found that the antibody amounted to roughly 40–70% of the increase in globulin, and stated: "That this increase in globulins is . . . closely connected with antibody production is indicated by several facts. The increase in globulin, though greater than the amount of antibody produced, still tends to parallel the content of antibody. . . Injection of gelatin which produces little or no detectable antibody, produces little or no increase in the various globulin fractions." Bjørneboe (12) found that in hyperimmunized rabbits (more than ten milligrams antibody nitrogen per cubic centimeter) all the globulin in the serum was antibody; in other words, all the "normal" globulin had been replaced by antibody.

It seems not impossible that the increase in γ globulins consequent on immunization, over and above what is known to be antibody, may be made up largely or perhaps completely of two additional fractions: *first* (the minor one?), antibody to other antigens to which the animal has previously responded—the "anamnestic phenomenon"—of which there are probably a great many, and *second*, antibody directed towards the antigens actually injected, but of such poor quality or avidity (see page 225) that it either fails to give any demonstrable reaction with the antigens, or is washed off the precipitate or "agglutinate" in the course of preparing the latter for analysis, and consequently escapes classification as "antibody." It is not impossible that this latter fraction is fully equal to or greater than the detected antibody in amount.

Finally there may be mentioned the study of Duran-Reynals (49) who found in chicken serum a factor which would flocculate saline extracts of the tissues of many animal species. This factor develops shortly after hatching; at the same time the "normal" antibodies such as bacterial agglutinins are developing, and the amount of the factor is increased by immunization of the chickens with any good antigen. He suggests that this flocculating factor may represent a first stage in the modification of (normal?) globulin and thus the lowest grade of antibody.

22. Valence of Antibodies

It is common to speak of a polyvalent serum, meaning one which contains antibodies for more than one antigen, e.g., against several types of *Pneumococcus*. The antibodies in such sera are separate and distinct.

There is another question of valence which is the one we wish to discuss here, namely, the number of specific combining groups on a single molecule of antibody.

It is hardly likely that the specific reactivity of an antibody molecule could be distributed uniformly over the whole surface, for what we know of specificity shows that orientation in space is important, and the combining group of an antibody must therefore possess an "up" and a "down," a "front" and a "back." Also, either of the theories of antibody formation outlined here would lead us to predict that a molecule of antibody could not have more than about two localized reactive groups. Some observations suggest that antibody does usually have two (or possibly sometimes more) combining groups. Ultracentrifugal studies of the toxin–antitoxin reaction by Pappenheimer, Lundgren, and Williams (143) suggest that the horse antitoxin molecule *may* be capable of combining with two molecules of toxin, and would thus possibly be divalent (1, 81, 153).

When slides are coated with a film of antibody about 45 Å. thick, antigen can be specifically deposited (62, 74, 152), on top of this another film of antibody, and finally another film of antigen. If the antibody molecules are all oriented alike in such films, this would seem to demonstrate that the antibody is at least divalent. However, a colleague has pointed out to the present writer that it is perhaps not excluded that such films might consist of molecules arranged alternately up and down, in a head–tails–head–tails . . . arrangement, so that even antibody possessing only one reactive site per molecule might still give this "sandwich" effect.

It has been observed in a number of cases (87, 95, 142) that in a serum there may be present antibody which has the power of combining with antigen, but does not form a precipitate. Such antibody has been variously referred to as low-grade, incomplete, imperfect. Heidelberger (81) calls it "univalent," but states elsewhere (95) that the word "univalent" is not used in the literal sense. The special properties of this antibody may perhaps be due to imperfect specificity (see page 36), or to physical properties different from those of "good" or "polyvalent" antibody, or to both (see Chapter VI, page 266). Wiener (187) apparently believes that certain such antibodies are univalent.

There is one theory (133) of antigen–antibody reactions which necessarily supposes them to be polyvalent, or at least divalent, but since we can not regard this theory as completely established, this does not prove the polyvalency of antibodies. The proper way to settle the point would seem to be by studying the molecular composition of antigen–antibody compounds made with excess antigen. Such studies as have been made thus far apparently have not revealed any compound containing more than two molecules of antigen in combination with one of antibody, so that the maximal valence so far found for antibody is two.

From certain data of Haurowitz, Kraus, and Marx (78), which led these authors to conclude that the antibody was monovalent, Pauling (145) concludes it was probably divalent. Pauling and collaborators (147) have calculated the valence of other antibodies from analytical studies on precipitates made with various haptens (see page 128). Objections can be raised against their experimental technic, however, (112) and also it is likely that many and perhaps most of the haptens used by Pauling are not molecularly dispersed in solution (10), or, in other words, they are aggregated, a state of affairs which we have known about, in the case of dyestuffs, for many years. If the haptens are aggregated, the valence of each particle of hapten is greater than computed from the simple chemical formula, and little reliance can be placed on Pauling's calculations of antibody valence.

The first attempt to study the question of antibody valence experimentally seems to have been the study of Haurowitz and Schwerin (79), who found that antibodies to an arsanilic acid-treated sheep globulin never contained combining groups for both the arsanilic acid hapten and the sheep protein in the same molecule, and concluded, therefore, that their antisera contained, mainly at least, univalent antibody molecules, thus not confirming the prediction of Pauling that antibodies possessing both combining groups should be found (see also Landsteiner, 120).

Landsteiner (120) mentions that special antibodies adjusted to antigens having, respectively, many or few haptenic groups do not seem to have been demonstrated. Since antibodies specially adjusted to highly multivalent antigens would presumably themselves be highly multivalent, this suggests that the valence of antibodies is low (meaning probably a valence of one or two).

Antigen, on the other hand, is almost always multivalent, and compounds have been observed in which over one hundred molecules of antibody were combined with one of antigen (135, 138).

23. MEASUREMENT OF CONCENTRATION

In view of the importance of antibodies in immunity, methods of determining them quantitatively have considerable interest.

In the early stages of immunology, when the chemical nature of antibodies was completely unknown, methods of determining them quantitatively could be only comparative, and even these were very crude. The essence of practically all these methods was to make successive dilutions of the serum and test for the antibody activity, recording the highest dilution which was still active. The test could be made through any one of the antibody's activities, agglutination or precipitation being commonly used. With precipitation, the technic of diluting the antigen and testing against a constant dilution of serum became common, probably because precipitating sera are not active at very high dilutions. The accuracy of such methods, even assuming that they really measured antibody, was clearly not very great. It was usual to make the dilutions successively double, so that there was automatically an error of 50 or 100% in the determination, since it is often difficult to choose which of two tubes represents the final active dilution. In addition, the dilutions were often not very accurately made, and small amounts of original material remaining in a pipet could change enormously the later dilutions.

There is also considerable uncertainty as to exactly what these methods measure. Particularly, the method of diluting the antigen can hardly test the amount of antibody except in a very rough way (120). It has been found by several workers that the limiting dilution (called by immunologists the *titer*, though this is strictly not a very accurate use of the word) giving precipitation was almost independent of antibody concentration (37, 100, 136, 166). However the method is rapid and easy to apply, and there is reason to think it does measure something about the power of a serum, possibly the "avidity," so it will probably be retained for some time, at any rate as a preliminary test. The method of diluting the serum is better, but still rather inaccurate. The reasons for this will be better understood after a consideration of the laws governing antigen-antibody reactions. Sometimes the method of limiting dilutions is the only one at our disposal to determine the strength of a serum (e.g., in some antivirus antisera), and is of course much better than nothing.

The methods developed by Ehrlich for measuring the potency of antitoxic sera constitute essentially a limiting dilution method. First an arbitrary unit of toxin, the amount sufficient to kill in four to five days a guinea pig weighing 250 g. (minimal lethal dose, MLD), was established. Most toxins contained many such units per cubic centimeter, so a "normal" solution was designated as one containing 100 such units per cubic centimeter. Then the amount of antitoxin needed to neutralize this was determined. Antitoxin proved to be more stable than toxin, so a certain dried antitoxic serum was set up as the reference standard. Then new batches of toxin were titrated against this.

Unexpected difficulty was encountered, because the quantitative course of serological reactions was at that time unknown, and it was considered puzzling when it was found that the amount of toxin which, after it had been mixed with one unit of antitoxin, left sufficient excess toxin free to kill a 250 g. guinea pig, was not just twice the amount which neutralized just one unit of antitoxin. Instead, many times this amount was required (Fig. 27). This led to a rather confusing set of theories about toxin–antitoxin reactions, which we need not go into here.

For such an important determination as antitoxic power, the determination of the neutralizing dose (L_0) was not accurate or definite enough, since there was no experiment giving a sharp end point, so it proved better and more reproducible to determine for each new toxin the amount which, when mixed with a unit of the preserved standard antiserum, would kill a 250 g. guinea pig in four to five days. Thus the L_+ dose, where the + was originally a cross signifying the death of the animal, was established. New batches of antitoxin could be standardized by reference to this temporarily standardized toxin, using the same end point. This is somewhat similar to the use of an intermediate unstable standard solution in volumetric titration, except that the end point in our case is not neutrality, but a sufficiently toxic mixture to kill.

The amount of an unknown antitoxic serum which gives the same amount of neutralization (allowing death of a guinea pig in four to five days) with the L_+ dose of toxin as does the standard antitoxin can be assumed to have the same curative power, allowing for variation between different guinea pigs. Such allowance can be made, and the accuracy of our estimates of the potency of the new serum can be judged, if statistical methods are applied to the planning and interpretation of the experiment. These methods are discussed in Chapter XI, where examples of such titrations are given.

Later Ramon (154) discovered that when toxin and antitoxin were mixed in the proper proportion they formed a precipitate (flocculated). From this observation he developed a method of titrating toxin and antitoxin *in vitro*. The technic of this reaction is discussed in Chapter XI, and the theory in Chapter VI.

Being a test tube method, this procedure is much more rapid and probably gives a better estimate of the actual amount (weight) of antibody present than does the Ehrlich titration. Because of variations in the quality ("avidity") of antibody in different lots, it may not necessarily give a better estimate of the protective power, if enough animals are used in the Ehrlich titration (which is not always done).

The method depends upon observation of the rates of flocculation of mixtures of toxin and antitoxin. The amount of toxin which gives most rapid flocculation with one standard unit of antitoxin is called the L_f dose. It is not usually the same as the L_+ dose determined by using animals, but is usually somewhat smaller. It has been found to represent 0.00042 to 0.00048 mg. toxin nitrogen, or some 30–40 MLD. Unknown sera are titrated against the freshly standardized toxin, using the same technic. This method is used in many laboratories for preliminary titration, but has not displaced the Ehrlich method for final standardization.

In 1926 a valuable observation of much the same sort was made by Dean and Webb (40). They found that with the system they were using (horse serum as antigen and rabbit anti-horse-serum antibody), mixtures with antigen and antibody in different proportions precipitated at different rates. It was found that the most rapid precipitation (flocculation) occurred, using any given concentration of antiserum, with one certain concentration of antigen. Tubes containing less than this amount of antigen went more slowly, as did tubes containing more. Irrespective of the absolute dilutions, the dilution of antigen in the fastest tube, divided by the dilution of antiserum, was a constant, which Dean and Webb called the point of optimal proportions. They found that, in their system, this tube contained a neutral mixture (i.e., neither antigen nor antibody, or only traces of both, in the supernatant fluid), after the precipitate had settled out. This is not always true of other systems, but precipitates made at the optimal proportions point contain antibody and antigen in a ratio which is roughly constant for any given system (18, 19). Most, and frequently all, of the antigen is precipitated at this point, so, knowing the amount of antigen added and the ratio of antibody to antigen for this system, we can calculate the amount of antibody in a measured amount of serum. Even without all of these data, a comparison of the strength of different sera is possible by determining their optimal proportions points against the same antigen. The procedure will be further discussed in the chapter on antigen–antibody reactions; the technic is given in Chapter XI.

Immunologists were baffled for some time by the problem of estimating the actual amount (weight) of antibody in an antiserum, as no method was known to distinguish the antibody chemically from the other constituents of the serum. Finally rough determinations were made by Landsteiner and Prášek (122), by precipitating with an anti-horse serum the agglutinin which could be split off from typhoid bacteria agglutinated by horse antityphoid serum. Methods capable of routine application were subsequently developed, based on the measurement of the size of a specific precipitate produced by the action of a precipitating serum on an antigen. If the amount of antigen used were known, and it could be assumed that it was all precipitated, the rest of the precipitate might be assumed provisionally to be antibody. This was denied by some workers, who spoke of a precipitate's carrying down large amounts of nonspecific proteins from the serum, but we now know that, if precipitates are washed before analysis, the assumption is true (to limits of analytical error). Even those who accepted the hypothesis, however, found it difficult to make good quantitative determinations of the amounts of antibody, as modern micro-chemical methods had not been developed. Attempts to make the determinations by weighing were tedious, and gave only fair accuracy. The estimation of the antibody and antigen by nitrogen determinations was made possible by the micro-Kjeldahl procedure devised by Parnas and Wagner. In the way of any extended study of the composition of specific precipitates, however, stood the difficulty of distinguishing the nitrogen due to the antibody in a specific precipitate from that due to the antigen.

The use of an antigen containing some special chemical characteristic by which it could be differentiated from the antibody, offered a way out of this difficulty (85, 192, 193). It then became possible to analyze the precipitate for nitrogen, thus getting an estimate of the total protein present, then to determine independently the amount of antigen, and by subtraction, the amount of antibody. If the precipitate were made in such a way as to obtain all the detectable antibody in the serum, one could thus estimate the actual weight of antibody protein per volume of serum. Heidelberger and Kabat (83) later showed that, if suitable blanks were done on the bacteria, it was also possible to determine by nitrogen analyses alone the amount of agglutinating antibody in a serum.

Since we have seen (p. 31) that the various antibody functions of an antiserum are often simply various manifestations of the activity of one and the same sort of antibody, it was reasonable to suppose that the agglutinating antibody and protective antibody, in a serum known to have protective action against the micro-organism used, might be one and the same, and consequently the amount of agglutinating antibody should be a good measure of the protective potency of the serum. This was found to be true for antipneumococcus sera, so far as could be judged from experiments designed to test it (92). Methods based on this fact have been extensively applied by Heidelberger and collaborators and by others (see 65, 81). Goodner, Horsfall, and Dubos (72) recommended them for standardizing antipneumococcus serum. Descriptions of the technic will be found in (65, 72, 83).

It has since been found that determinations of the precipitating antibody from antipneumococcus sera, by analyses for nitrogen of precipitates made with the specific carbohydrates, provide an even more convenient measure of the potency of the serum (65). Apparently this method is even more accurate than the Heidelberger and Kabat (83) method, for Gerlough, Palmer, and Blumenthal (65) report that the precipitin seems to be identical with the protective antibody, whereas some of the agglutinin present does not have any appreciable protective function. These authors suggest the following values as the approximate number of mouse-protective units in one milligram of antibody nitrogen: type *2*, 900 units per milligram of nitrogen; types *1*, *4*, and *8*, 1100 units; types *3* and *5*, 1500 units; and type *7*, 2000 units.

Heidelberger and Treffers (93) have estimated the total hemolysin in lytic sera by determining the nitrogen added to sheep stromata suspensions when treated with lysin. Henriksen and Heidelberger (97) have applied a quantitative agglutination procedure to the determination of the amount of antibody in antisera to hemolytic streptococci.

SUMMARY

(1) Immunology is essentially a practical subject, concerned with preventing disease or influencing its course. As treated here, however, it does not include subjects such as bacteriology, public health, or chemotherapy. *(2)* Since immunity is closely bound up with certain properties of the blood, the chemical constitution of blood, plasma, and serum is discussed. Plasma is blood minus cells. Serum is plasma minus fibrinogen and other substances concerned in clotting. *(3)* Antibodies are blood proteins of the class called globulins. *(4)* It seems that one and the same antibody may manifest itself in more than one way, as by agglutination, precipitation, etc. ("uni-

tarian" hypothesis). However there is evidence that a single pure antigen may on occasion give rise to more than one antibody. (*5*) Antibodies may react with the antigen so as to precipitate, agglutinate, dissolve (lyse), kill, cause swelling, etc. The reaction may involve another set of blood constituents collectively called complement. (*6*) Antibodies are more or less specific. Specificity has been defined by Landsteiner as "the disproportional action of a number of similar agents on a variety of related substrata." Specificity is chemical in basis. (*7*) The antibodies in a given serum do not usually seem to be entirely homogeneous. Antibodies in the same serum may be directed exclusively against some one part of the antigen, or simultaneously against more than one, and the sharpness of their specificity may vary. Specificity of antibodies tends to become less as immunization is prolonged. (*8*) "Normal antibodies," i.e., antibodies which react with antigens with which the host has not, so far as we know, come in contact, are sometimes found. The correspondence may be accidental in some cases, and the specificity not of a high order; in other cases, such as the isohemagglutinins anti-A and anti-B, the specificity is good but we do not know why the antibody occurs. Some "normal antibodies" are doubtless the result of subclinical infections. (*9*) The chemical nature of antibodies is in general that of normal serum globulins, although occasionally physical or chemical differences may be detected. Antibodies are heat labile, denaturable, precipitable by protein precipitants, digested by enzymes (although perhaps sometimes more resistant than normal proteins to certain enzymes). (*10*) The molecular weights of antibodies have been determined in many cases by the ultracentrifuge. They are usually the same as some of the normal serum globulins, ranging probably from about 160,000 to 1,000,000. (*11*) Antibody molecules, again like normal serum globulins, seem to be asymmetrical. (*12*) The electrophoretic mobilities of antibodies, as determined in the Tiselius apparatus, place them usually in the γ globulin class, with few exceptions. (*13*) Chemical analyses do not differentiate antibodies from normal γ globulins. (*14*) There is a rough parallelism between solubility and electrophoretic mobility at the usual *p*H. (*15*) Antibodies, even to the same antigen, display differences when they come from different species. Some species seem to be definitely poorer producers of precipitins than the rabbit. The horse produces two different types of antibody, with different molecular weights. Rabbit antipneumococcus antibodies seem to have a number of advantages over horse antipneumococcus antibodies. (*16*) Some antibodies differ physically or chemically from the normal globulins present and can be separated from them by utilizing this fact. Felton's purification of horse antipneumococcus antibody is an example. Generally the differences are not great enough to make this possible, and purification is achieved by dissociating (as by salt solutions) antibody from a washed antibody–antigen precipitate. Enzymes have been found of assistance in some instances. Northrop obtained in this way crystalline diphtheria antitoxin, although it had probably undergone some alteration. (*17*) Antibodies may be formed in the cells of the reticulo-endothelial system, but are also found in the lymphocytes. Evidence includes results of extirpation of certain organs, "blockade," use of colored or otherwise detectable antigens, etc. Possibly almost any kind of body cell can produce some antibodes, for local concentrations have been observed. (*18*) An interval of several days usually elapses after the introduction of the antigen before antibody appears suddenly in the circulation. The antibody concentration gradually declines, but requires a long time to reach zero. This is important clinically. Passively introduced antibody disappears rapidly from the circulation because it is not replenished. (*19*) An antibody which the tissues once manufacture will reappear more rapidly if a new dose of the same (or a related) antigen is given. This is called the anamnestic ("recollection") reaction. Some have even claimed that a dose of quite unrelated antigens had some such effect. (*20*) Theories of antibody formation were proposed independently by Breinl and Haurowitz, Alexander, and Mudd. They suppose that serum globulin molecules, as they are being formed, are influenced in their chemical configuration by the presence in the cell of a molecule of antigen, and thus are laid down with part of their surfaces corresponding, in reverse, to part of the antigen. One molecule of antigen might influence the formation of several successively formed antibody molecules. Pauling believes that only the configuration assumed by the ends of the protein polypeptide chain when it folds up is altered by the presence of the antigen. On the basis of this theory he attempted to make antibodies *in vitro*, although other investigators had failed in such attempts. Confirmation of his claims of success is still lacking. Burnet does not think there is any good reason to believe that the mere presence of a molecule of antigen would cause newly synthesized globulins to have a complementary (antibody) configuration, but thinks it more likely that certain enzymes become modified in breaking down the antigen particles and as a result synthesize antibody. No one of these hypotheses can be regarded as finally established. *(21)* It seems possible that all serum γ globulin may be antibody of some sort. Antibodies may be found at times in the α or β globulins, but probably these globulins primarily have other functions. Hyperimmune animals have been observed in which the only globulin present was antibody. *(22)* The number of specific combining groups on an antibody molecule is called its valence. Antibodies seem to be univalent or divalent, although antibodies of higher valences have been postulated. Antigens are apparently always polyvalent. *(23)* Determination of the highest active dilution of an antiserum may serve to estimate the antibody content roughly, and is often used. This may be done in test tubes or in animals (Ehrlich, etc.). It is more doubtful what determination of the limiting reactive dilution of the antigen means. Determination of flocculation optima (Ramon, Dean and Webb) in the precipitin test is probably more accurate. Modern microchemical analytical methods enable the antibody content of a serum to be determined with greater precision (Heidelberger and followers). If the antigen contains no nitrogen or contains a special "marker," micro-Kjeldahl determinations give an estimate of the antibody present.

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Chapter II

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CHAPTER III

ANTIGENS

Divisions into sections of a subject like immunology are always more or less arbitrary, and a strictly logical order of presentation hardly seems possible. It will not have escaped the attention of the reader that we have already said a good deal about antigens before coming to the present chapter. In this and succeeding chapters, in turn, we shall have to mention topics theoretically reserved for later treatment. It is hoped that this procedure, though perhaps regrettable from the point of view of system, will on the whole improve the clarity of the presentation, rather than the reverse, and help to make the subject a more integrated whole in the mind of the reader.

The word "antigen" has been used in two senses, first to denote a substance which, when introduced parenterally* into an animal, will cause the production of antibodies; second, to denote a substance which reacts in a visible way with antibodies. The latter class includes practically all of the first, but the reverse is not so; that is, there are a number of substances which, although they react with antibodies, have not been shown to produce antibodies when injected into animals. A substance is not said to be *antigenic* unless it will cause the production of antibodies.

1. Conditions of Antigenicity

A very large number of substances have been tested for antigenic power, and it is natural to consider them as a group and try to see what characteristics they have in common. Unfortunately the knowledge which has been acquired thus is very meager; we are not yet able to say from the chemical properties of a substance that it will *certainly* be antigenic or nonantigenic. However, certain general facts do emerge.

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* That is, outside the digestive tract, although antibodies may sometimes be produced following the ingestion of antigen In such cases presumably some is absorbed before digestion begins, or at least before it is complete.

(*a*) Antigenic substances are always large molecules, of molecular weight of the order of 10,000 or higher (excluding simple compounds which have been found to *sensitize*; see Chapter VIII), and consequently are usually colloidal in solution. Correlated with the large molecular size is the inability of the antigenic substances to go through the walls of veins, collodion, or cellophane membranes which pass smaller molecules such as sugar. There is some indication that, in general, the larger the molecule the better the antigenic power (98). The high antigenicity of hemocyanins and tobacco mosaic virus is in line with this.

The importance of particle size in antigenicity is indicated by observations that nonantigenic or weakly antigenic substances can sometimes be made antigenic by adsorbing them on particulate matter such as collodion particles, kaolin, or charcoal (60, 101, 148, 161). Even merely suspending particulate material, especially killed microorganisms, in a solution of an antigen may suffice to enhance the antigenic stimulus so that fair antisera may be obtained against poor antigens such as hemoglobin (17). Also, it may be remarked that Mudd and Wiener (128) found the intact streptococcus cell to be of much higher antigenicity than the isolated purified M protein.

The use of adjuvants to obtain an enhanced antibody response in immunisation is becoming more and more popular. In particular the procedure introduced by Freund and McDermott (48) of incorporating the antigen in a lanolin-like substance (Aquaphor or Falba), which is then emulsified with a suspension of tubercle bacilli or other acid-fast organisms, is fairly widely used (49, 88).

(*b*) The majority of antigenic substances are proteins, and it was formerly believed that only proteins could act as antigens. It is now known that some lipides, particularly cholesterol and related compounds (98), when injected mixed with antigenic protein, cause the production of antibodies. Purified specific capsular polysaccharides of the pneumococcus are devoid of antigenicity for rabbits and horses, but they can, under proper conditions, call forth the production of specific antibodies in a mouse or a man (35). Lipide-carbohydrate compounds, even when carefully purified to remove protein, will produce antibodies when injected (3, 9, 43, 44, 46). Certain substances belonging to this latter class are hardly inferior to proteins as antibody producers. Aside from these three, it has not been definitely established that any other class of compound can be antigenic.

Not all proteins are equally good antigens. Here, and in much that follows, we mean "antigens for rabbits." This is the animal most often used. Our knowledge of others is less; some species seem definitely inferior to the rabbit as precipitin producers. Even within this species individual differences may be found in antibody-producing power for a given antigen. Probably the best antigens are complex mixtures, such as whole blood serum, or bacterial or blood cells. Next, perhaps, come certain proteins, such as hemocyanins and ovalbumins. Hemoglobin is a poor antigen and has in fact several times been erroneously reported to be nonantigenic. Finally gelatin (a derived protein) is nonantigenic, except as a conjugate (see page 98). The simplest proteins, the protamines, are also nonantigenic (166).

It has been proposed that the nonantigenicity of gelatin is due to the relatively rapid elimination of this protein from the circulation (67). This same characteristic seems to be the cause of the relative inefficiency of gelatin as a blood substitute.

(c) As a rule, an animal's own proteins, or those of another individual of the same species, are not antigenic for it. In immunology this principle has received the name of *horror autotoxicus* (fear of poisoning one's self), which is stated thus: an animal will never produce antibodies to any substance normally found in his own circulation.

Some workers apparently still believe that antibodies to an individual's own antigens may be produced, but never observed, since they would continually be removed by combination with the big excess of the antigen constantly present. Others feel that this violates some sort of "principle of laziness" in nature, and that such antibodies are probably not produced at all.

The principle as stated above is qualified by the restriction that it applies only to substances in the animal's circulation. This qualification is needed, for it has been found that protein from the lens of the eye (see 166) will produce antibodies in animals of the same species (see 92, 118), and it has been claimed (see 114) that a guinea pig can be sensitized with the lens of one eye and later shocked anaphylactically with the lens of the other. Lewis (114) found that lactating goats could be caused to form antibodies to their own casein. These antibodies reacted to an equal degree with cow casein. Hektoen (cited in 114) reported obtaining other *isoantibodies*, in rabbits, against thyroglobulin (the iodine-containing protein of the thyroid gland) and fibrinogen from the blood, although Stokinger and Heidelberger (158) later found definite differences in their antisera to thyroglobulin from man, hog, cow, and sheep. Thus it seems established that certain proteins of the body which never normally come into the circulation can act as "isoantigens" (antigens for individuals of the same species as the individual producing them). Lewis (116) found that the tissue lipide extracted by alcohol from the brain was isoantigenic when injected mixed with foreign serum.

From the above facts we may venture to surmise that: (*1*) the antigenic power of proteins is greater, the more removed is their source from the experimental animal in the zoological scale; (*2*) very simple proteins are not antigenic, perhaps because they disappear from the circulation before enough can be fixed by the antibody-forming cells. These guesses may not be wholly correct, of course. Plants are much further removed from rabbits in the taxonomic scale than are other animal species: principle (*1*) might lead us to predict that plant proteins would therefore be much better antigens than animal proteins. There does not seem to be any particularly convincing evidence that this is so, although the plant proteins which have been studied do seem to be good antigens.

Gelatin contains no tyrosine or tryptophan, and but little phenylalanine. Its nonantigenicity led Obermayer and Pick (see 95, 166) to infer that the aromatic radicals of the protein molecule are of particular importance in determining antigenic activity. In support of this idea Wells (166) mentioned several facts, among them the observation of Obermayer and Pick that substitution in the tyrosine nucleus (see p. 96) markedly alters the immunological specificity of proteins. It is difficult to test the idea, but there are some observations rather contrary to it. It has since been found that substitution in other parts of the protein molecule also alters the specificity. Also, aromatic groups can be introduced into gelatin without making it antigenic (82). Landsteiner points out that not all proteins possessing aromatic groups are antigenic; examples of this are proteins which have been altered sufficiently by acid or alkali. In other experiments (27, 76) it is true that introduced aromatic groups did make gelatin antigenic, but only feebly so; in both of these cases the observation was made that the antisera against the gelatin compound sometimes failed to react with it, but did react with other, more antigenic, proteins which had been subjected to the same chemical treatment. Hooker and Boyd concluded that their experiment (76) did not show that the nonantigenicity of gelatin was due solely to its deficiency in aromatic amino acids, since the possibility that nonaromatic prosthetic groups, especially if they contained polar groups, might also make gelatin antigenic, had not been tested.

Some carbohydrates, not possessing aromatic nuclei, are nevertheless somewhat antigenic, and the "Boivin antigens" (see p. 127) are highly so. This, together with the above facts, suggests that the power to immunize depends on general properties of the molecule, or, though this is much less likely, on some specific characteristics as yet unknown to us which all antigens have in common. In any case, the possession of aromatic groups does not appear to be one of these characteristics.

Finally, Landsteiner's observations—that simple substances not causing antibody formation, and not necessarily containing aromatic groups, can still react specifically with antibodies—were rather against the aromatic determinant idea, which was partly based on the notion that antigenic capacity and ability to react with antibodies were inseparable.

When proteins are racemized by treatment with alkali, they are no longer antigenic. Since it was found by Dakin and Dudley (32) that such proteins were no longer susceptible to hydrolysis by enzymes, it was suggested that these facts were causally related (159). This suggestion recalls the "digestion" hypothesis of antibody formation (see Chapter II). However other workers have more recently found (117) that these racemized proteins are not completely resistant to enzyme action. It has also been found that the antigenicity is lost more rapidly than the optical activity (171). Landsteiner (95) has pointed out that the alkali might destroy structures significant for the antigenic function at the same time it produced racemization. The lower antigenicity of alkali-treated proteins may possibly be connected also with the smaller size of their molecules; Boyd (11) found that on treatment of crystalline ovalbumin with alkali there was at first a marked rise in optical activity to several times the initial value, followed by a slower fall to a minimum, indicating possibly that the molecule was first split, then racemized.

That racemization does not in itself mean nonantigenicity is indicated by the observation of Landsteiner and Barron (99) that nitration of racemized proteins restored some degree of antigenicity, as, to a less extent, did iodination (87).

It does not, therefore, seem to have been proved that optical activity is essential to antigenicity, although optical activity may play an important role in specificity.

2. Competition of Antigens

When a weak and a strong antigen are injected together, the animal may fail to respond to the weaker antigen, particularly if this is present in smaller amount (98). This (probably rare) phenomenon has been termed "competition of antigens." It is roughly the converse of the effect obtained with adjuvants which are good antigens (see above).

3. The Specificity of Antigens

The specificity of antigens resides in the structural peculiarities of their molecules. While it has not been possible to correlate immunological differences in substances, particularly proteins, with their chemical structure in every case, there is no doubt that this is simply because of our ignorance of the detailed structure of these compounds.

The dependence of specificity on chemical structure is proved by several lines of evidence. (*a*) Purified proteins that exhibit chemical differences can nearly always be differentiated serologically (31, 33, 62, 69, 79, 112, 116); (*b*) carbohydrates related structurally give serological cross reactions (4, 54, 58); (*c*) simple chemical substances (haptens and some allergens) give cross reactions when chemically similar (95); (*d*) chemical alteration of antigens generally alters their specificity; (*e*) corresponding proteins of different species which are functionally, and thus probably structurally, related generally cross-react.

The very large number of specifically different antigens need cause no surprise, in view of the power of the antibody-forming mechanism to recognize the smallest shades of chemical difference (see p. 112) and in view of the enormous number of different proteins that can be built up from the known amino acids and the smaller but still enormous number of possible polysaccharides (68, 172).

4. Location and Size of Specific Groups

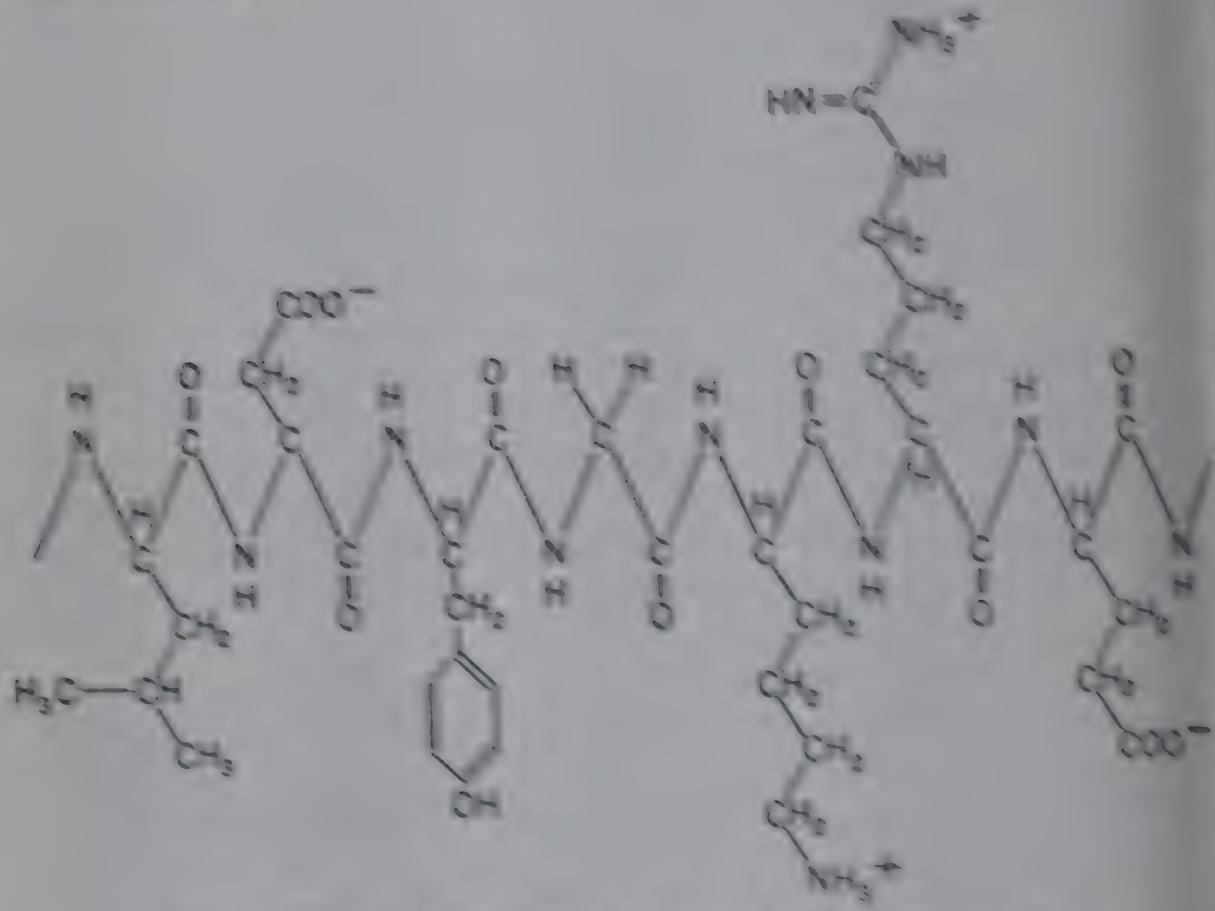
Antibodies can be obtained which are specific for a simple chemical compound (called in this connection a hapten) and which precipitate proteins which have had this group introduced into them. In this case the reactive group in the antigen is of known size, i.e., it equals the introduced hapten (except in cases where the adjacent portions of the protein molecule seem also to play some role). The location and spacing of the haptens in the conjugated protein molecule, however, remain unknown.

With the natural protein antigens we do not have even this information to guide us. It is hardly conceivable that the whole molecule serves as a determinant; if either of the theories of antibody formation described in Chapter II is correct, this is impossible. Attempts have been made to get some idea of the size of antigenic determinants in proteins by testing for inhibiting power the breakdown products resulting from the action of enzymes, etc., on proteins (100, 108), but without success. A reason for the difficulty may be that in the protein the determinant might possibly be constrained by intramolecular forces to take up a configuration somewhat different from that which it assumes when separate.

Landsteiner (97) reported obtaining hydrolysis products of silk, consisting of peptides having molecular weights of about 600 to 1000, which were capable of inhibiting the reactions of precipitin sera for silk. This experiment may perhaps serve provisionally to set an upper limit to the size of the antigenic determinants in natural proteins. It must be recalled, however, that compounds simpler than the actual determinant, but chemically related to it, have been observed to inhibit, in studies on conjugated antigens (page 441). It is therefore possible that the complete determinant in the silk molecule could be even larger than this experiment indicates. This does not seem very likely, however.

Another reason for thinking that a determinant in a protein must be considerably less extensive than the whole molecule is the number of molecules of antibody (about six for ovalbumin, over one hundred for some hemocyanins) which may unite simultaneously with one molecule of antigen (see Chapter VI), indicating that at least that many determinant groups are present (see page 243). One thing is practically certain: the specific determinants in natural proteins are combinations and arrangements of amino acid residues. Though carbohydrates or metal-containing prosthetic groups as in hemoglobin may be present, no case has thus far been studied in which the specificity was determined by them.

We know proteins to be made up of chains of amino acids, held together by the peptide linkage. If such a chain were stretched out, part of it might look like Figure 10. As proteins are present in solution, they are globular or ellipsoidal in shape, so we must assume the peptide chain is rolled up. Marrack (120) suggests that, considering the importance of the terminal amino acid in the peptide haptens studied by Landsteiner and van der Scheer (see Table VIII), it is probable that the immunological character of natural proteins is determined by the arrangement of amino acids on the surface of the molecule. This is supported by evidence that antigens react as spherical or ellipsoidal molecules (14, 16, 139) (see Chapter VI). It is also compatible with results (22, 152) indicating that monofilms of proteins about 9 Å. thick still possess the power of reacting specifically with antibody.


NH_3^+
HN=C
NH
CH_2
CH_2
CH_2
COO^-
CH_2
H
O
H
N
C
C
C
N
H
C
N
C
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CH_2
H
O
CH_2
H
O
CH_2
H_3C—CH
CH_3
CH_2
CH_2
CH_2
CH_2
COO^-
OH
CH_2
NH_3^+

Fig. 10. Part of the polypeptide chain of a hypothetical protein.

Marrack further supposes that several amino acid residues together may form an "active patch," with a characteristic spatial distribution of forces, on the surface of the protein molecule. He points out that such a patch might be more or less completely altered by the molecular rearrangements taking place on denaturation; the latter, as we know, affects specificity and antigenicity.

Bergmann proposed the hypothesis (see 7) that ". . . in every protein each amino acid residue is distributed throughout the entire peptide chain at constant intervals, i.e., each amino acid residue recurs with a characteristic whole number frequency. . . The protein molecule therefore contains a great number of superimposed frequencies." Marrack pointed out (120) that if the amino acids are arranged in a regular fashion "there must be nodes or patches in which certain amino-acids recur together... It may be supposed that such localized recurring groups of amino-acids form the determinant groups of natural proteins..."

The ultimate fate of Bergmann's hypothesis remains to be seen. Some of the modern analytical work on proteins, such as that of Chibnall, does not seem to support it very strongly. If we accept it provisionally, it must be admitted that Marrack's suggestion has plausibility.

5. NUMBER OF REACTIVE GROUPS (VALENCE) IN AN ANTIGEN MOLECULE

Since we do not know the exact nature of the determinants in natural proteins, we can not determine accurately how many antigenic determinants are present in a single molecule. From analyses of precipitates for antibody and antigen, however, it is possible to determine the maximal number of antibody molecules which can combine with a molecule of antigen; the "valence" of the antigen can not be less than this. We thus set a lower limit for the valence of the antigen molecule. The following minimal values have been obtained (references in 81):

Antigen	Valence	Molecular weight
Ovalbumin	5	40,500
Serum albumin	6	70,200
Diphtheria toxin	8	70,000
Thyroglobulin	40	650,000
Busycon hemocyanin	74	6,760,000
Viviparus hemocyanin	231	6,630,000

Since the amount of antibody combining with a molecule of antigen tends to depend on the molecular weight of the antigen (14, 16), the results for other antigens of the same molecular weights as those in the above table would probably be of the same order of magnitude.

These results are clearly limiting values, or minimal valences, because it is conceivable, and even likely, that there are more reactive groups on the surface of an antigen molecule than can simultaneously be combined with, because the molecules of antibody get in their own way, so to speak (steric hindrance). Also, calculations from the data indicate that when antibody is in excess the surface of each molecule of antigen is completely covered by molecules of antibody (14, 16). Unless we are to suppose the reactive groups which are present are distributed in each case with exactly the right spacing between them to allow all to react before steric hindrance blocks them off, it would seem that there must be more reactive groups present than this minimal number.

In the case of artificial antigens, it is possible to estimate the number of active groups in a molecule of antigen by chemical analysis. This method was applied by Hooker and Boyd (76) to compounds of diazotized arsanilic acid and casein. It was found that preparations containing less than about 13 introduced groups per molecule would not precipitate with the anti-arsanilic antiserum. Similar results were obtained by Haurowitz (64), who showed that between ten and twenty introduced groups were needed, and furthermore that the failure of compounds of lower arsenic content to precipitate was not due merely to lessened acidic character. Compounds containing fewer introduced groups, down to only one per molecule, were found still able to immunize.

The results obtained with artificial antigens, contrary to those for natural proteins, may give too high a value for the valence, as we can not be sure that all the introduced groups are on the surface, or otherwise in a position to combine with antibody. So the actual number functioning may be less than indicated. Taking account of all these points, we may perhaps surmise that the valence of protein molecules of moderate size is somewhere between five and fifteen.

Certain nonprotein antigens, such as the pneumococcus polysaccharides, which are polymers of simpler compounds such as aldobionic acid, may contain the combining group repeated many times and are undoubtedly multivalent (120). In some cases we know the size and structure of the determinant groups, but still the exact number of combining groups per molecule can not be stated, as in most cases we do not know the molecular weight of these substances.

6. Effect of Chemical Alteration and Conjugation on Specificity

Since our knowledge of proteins is still quite limited, not as much has been learned about the nature of specificity by the study of the natural proteins as by the study of chemically altered proteins, haptens, and serologically reactive carbohydrates.

Almost any decided chemical change in a protein alters its specificity. It is worth while to survey the various methods which have been used in the study of immunological specificity. These methods may be conveniently considered as falling into two classes: alterations of the protein structure; and coupling of chemical compounds to the protein molecule (conjugation). We may define conjugated antigens as those giving rise to antibodies capable of reacting with the introduced group by itself. This permits in theory a sharp separation of the two types of antigens, although in some cases the requisite information is not available. Conjugation, of course, is always accompanied by some alteration of the protein molecule itself. However, this is relatively unimportant, as the tests are usually carried out in a way that eliminates any influence of the protein carrier. The advantage of conjugated antigens for our purpose lies in the fact that we know the chemical constitution of the reacting groups and the number of them per molecule of protein.

Alteration

Denaturation. The word denaturation is used to cover a number of mild alterations in proteins, not certainly related in nature, the common feature of which is a complete loss of solubility, at the isoelectric point, in water and in dilute salt solutions. The process was once considered irreversible, but is now thought by some to be reversible in some cases (1). Proteins can be denatured by strong acids and alkalis, salts of the heavy metals, light, heat, shaking, pressure, adsorption on a surface, and by the action of alcohol, acetone, and substances such as ether and urea. Some believe that there are degrees of denaturation (132).

Denatured proteins may fail to precipitate with antisera to the native protein, or may still precipitate somewhat, depending on the extent of the denaturation. It is an interesting fact that a denatured protein has been found to inhibit the formation of a precipitate by the natural protein and its antiserum, although it itself would no longer precipitate (135, 157). This suggests that perhaps some denatured proteins may still be able to react with the antibody, but the reaction for some reason does not form a precipitate. It has also been found that injection of a denatured protein may produce antibodies reacting with native as well as denatured protein (124).

Heating most proteins in solution soon destroys their power to react with their antibodies (casein is resistant to heating to 100°C). But *antigenicity* is not lost, for heated proteins will often produce antibodies when injected. The interesting observation has been made (51) that precipitins for heated proteins will often react with heated proteins of other species. Umesumi (163) even reported producing precipitins by injecting rabbits with rabbit serum which had been heated to 120°.

Oxidation. By oxidising proteins with potassium permanganate, products have been obtained which would immunise; and antibodies were obtained which precipitated the antigen injected, but not other proteins so treated, and not the untreated protein (see 95). Thus species specificity seemed to be preserved, although the immunological nature of the protein was completely altered.

Reduction. Blumenthal (8) found some diminution in the reactivity of serum albumin, but not egg albumin, after reduction with thioglycolic acid.

Digestion. Destruction of protein antigens by digestion of course results in a complete loss of their specificity. Pepsin generally causes a protein to lose rapidly the power of precipitating with antisera for the unaltered protein, although Parfentjev (see Chapter II) has found that antitoxin may retain its antibody activity after treatment with pepsin under appropriate conditions. If a peptic metaprotein is injected, antibodies may be obtained which precipitate the metaprotein and also the unaltered protein (see 95).

Acid and Alkali. Acids and alkalis react with proteins to form acid and alkali metaproteins. This decreases the antigenic activity, alkalis being much more active in this way than acids. Acid-treated proteins seem to lose their species specificity to some degree, and acquire the capacity of reacting with antisera for other such proteins (95).

Deamination. Lewis (113) found that treatment of casein with 7% acetic acid and sodium nitrite, a procedure which removed all free amino groups, did not change the more obvious antigenic characteristics of the protein. A yellow, difficultly soluble product was obtained.

Substitution Reactions; Formaldehyde. Formaldehyde reacts with the amino groups of amino acids, and probably with those of proteins, rendering them less basic. This fact is made use of in the formol titration of Sørensen. The reactions are discussed in a review by French and Edsall (47). Von Eisler and Löwenstein (38) and Landsteiner and Lampl (102) found that the species specificity of formaldehyde-treated proteins was not much affected. Horsfall

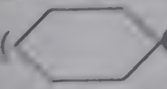
(84), using a somewhat different mode of preparing the antigens, found some cross reaction with formolized proteins of other species. Jacobs and Sommers (86) suggested that the unusual precipitability of Horsfall's preparations was due to other changes in addition to combination with the amino groups. They themselves found serologically demonstrable changes only in material from related species, resembling the alterations due to heat more than the more marked changes following halogenation or nitration. They report that very faint traces of turbidity in the test antigens may be associated in these experiments with false positive reactions. The preparation of toxoids by use of formaldehyde is discussed on page 407.

Esterification. Landsteiner (95) employed two methods of esterification, treatment of the protein with acid in alcoholic solution and treatment with diazomethane (CH_2N_2). The first method was intended to esterify the carboxyl groups, and the second, in addition to esterifying these, also methylated, on intensive treatment, the hydroxyl, amino, and imino groups. The products were insoluble and had to be tested for serological reactivity by the complement fixation technic. It was found that these esterified proteins had lost their capacity to react with antibodies to the unchanged proteins.

Acylation. Proteins are easily acetylated with acetic anhydride, $(CH_3CO)_2O$. The behavior of such products is similar to that of the methylated proteins. Landsteiner (95) concluded that the amino and hydroxyl groups are the ones affected. Some cross reaction was found between proteins containing different acyl groups.

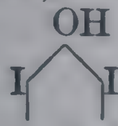
It has been found that the type-specific polysaccharide (S1) of type 1 pneumococci is acetylated in the form in which it occurs in the organisms. The preparations first isolated had been deacetylated by the use of alkali, with the loss of some antigenic properties. It was also found that the deacetylated polysaccharide would not absorb all the protective antibodies from antiserum produced by injecting whole cocci, whereas the acetylated form would (3).

Ketene gas, $CH_2:C:O$, may be used to acetylate proteins.

Benzoyl groups (CO—) have been introduced into proteins by Medveczky and Uhrovits (122) by treatment with benzoyl chloride. They suppose that the benzoyl groups go first on the carboxyl groups, then on the amino, and finally on the imino and hydroxyl groups. As before, the antigens acquire a new common specificity.

By the action of carbobenzoxy chloride (benzyl chloroformate),

$C_6H_5CH_2OCOCl$, Gaunt and Wormall (52) produced carbobenzoxyproteins. The reaction appears to be concerned mainly with the free amino groups. The original species specificity was almost completely destroyed, and the antisera reacted with other proteins similarly treated. The reactions were completely inhibited by carbobenzoxyamino acids, indicating that the specific group is $—NHCOOCH_2C_6H_5$. Phenylcarbamidoamino acids, containing the related group $—CHNHCONHC_6H_5$ also inhibited to a less degree.

Halogenation. Following pioneer work of Obermayer and Pick, iodine and bromine have been introduced into proteins. For a review see Mutsaars (130). The introduction of chlorine has been attempted; for references see Wormall (170). Iodinated proteins acquire a new specificity, and antisera to them react with other iodoproteins. The brominated proteins are only slightly different from the iodinated proteins, and cross-react with them. Wormall (170) found that 3,5-di-iodo- or dibromotyrosine, and to a less extent dichlorotyrosine, would inhibit the reaction of iodoprotein with its antibody. Halogenated imidazole was apparently not tried. Snapper and Grunbaum (155) found that any substance, including thyroxine, containing the 3,5-di-iodo-4-hydroxy group, , is capable of this inhibiting reaction. Kleczkowski (91) studied quantitatively iodination and its effect on the serological properties of horse serum globulin. He found that the ability to react with and to produce antibodies to native globulin disappeared almost simultaneously when the Folin color value fell to about 30% of the original value, i.e., when all the tyrosine was presumably substituted. He calculated that the amount of iodine which entered the molecule (11%) was all accounted for by the tyrosine groups, so there was no reason to suspect other alterations in the molecule. If this is so, we may suppose that tyrosine forms an essential part of the antigenic determinants of native horse serum globulin.

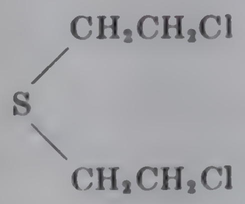
Nitration. Nitrated proteins, first studied serologically by Obermayer and Pick (see 95), have been investigated by several workers. Nitric acid has usually been used for the nitration; Wormall (170) also used tetranitromethane. The nitroproteins (xanthoproteins) are yellow and have acquired another new common property, for they cross-react serologically. The chemical change is probably the production of mononitrotyrosyl and nitrotryptophanyl groups.

By the method of specific inhibition Mutsaars (130) demonstrated that the serological specificity of the xanthoproteins is due to the nitrotyrosyl group. By testing numerous compounds, he found that the reactivity depends on the presence of nitro and hydroxyl substituents in the benzene ring and a carboxyl group which can be free or esterified. Boyd (11) and Mutsaars (130) found that the nitration of gelatin did not make it antigenic; according to the latter it does not precipitate with immune sera to other nitroproteins, but inhibits specifically.

In the case of methylated, acylated, halogenated, and nitrated proteins the species specificity is not entirely destroyed, as can be demonstrated by testing with diminishing quantities of antibodies, whereupon it is observed that the reactions are stronger with the homologous protein (95).

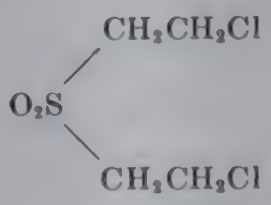
Diazotization. Proteins do not contain aromatic amino groups, but if they are treated with nitrous acid intensely yellow products are formed, which are almost indistinguishable serologically from the nitroproteins (104). It is supposed (see 95, 170) that the nitrous acid treatment results in the introduction of a diazo group into the tyrosine nucleus, and that the intense yellow color is due to the change of the benzene ring of the tyrosine residue to a quinoid structure. This is also thought to explain the color of the nitro derivatives. This would offer a reasonable explanation of the close similarity between the two classes of compounds. The serological similarity of the nitro- and diazoproteins has been confirmed in the case of gelatin by inhibition tests (129).

Treatment with Mustard Gas. Berenblum and Wormall (5) treated proteins with mustard gas (I) and with the corresponding sulfone (II):



$$\mathrm{S}\begin{array}{l}\diagup\ \mathrm{CH_2CH_2Cl}\\ \diagdown\ \mathrm{CH_2CH_2Cl}\end{array}$$

(I)



$$\mathrm{O_2S}\begin{array}{l}\diagup\ \mathrm{CH_2CH_2Cl}\\ \diagdown\ \mathrm{CH_2CH_2Cl}\end{array}$$

(II)

In each case a new specificity was conferred, although it was not as striking as in some of the above experiments. The two classes of compounds did not cross-react. Species specificity was retained to a considerable degree, but there may have been some unchanged protein in the preparations. It is thought that the reaction may involve the free amino groups of the protein.

Treatment with Phenyl Isocyanate. Hopkins and Wormall (82) produced phenylureidoproteins by treatment with phenyl isocyanate ($C_6H_5N:C:O$). The introduced groups go mainly, if not entirely, to the free amino groups of the lysine, as follows:

$$—CH_2NH_2 + O:C:NC_6H_5 \xrightarrow{pH\ 8} —CH_2NHCONHC_6H_5$$

Although not all the amino groups were substituted, cross reactions with the untreated protein were found to be much reduced, and a new specificity was conferred. The reactions could be inhibited by compounds of phenyl isocyanate with lysine or ε-amino-*n*-hexoic acid; compounds of other amino acids were less effective. The antisera reacted weakly with compounds prepared by treating proteins (including gelatin) with diazotized aniline presumably because of the similarity of the groups $—CONHC_6H_5$ and $—N:NC_6H_5$. Injection of the gelatin compound did not produce any antibodies which could be detected The *p*-bromo compounds were also studied, with similar results.

Treatment with β-Naphthoquinone Sulfonate. Fujio (50) reported that injection of proteins treated with this substance, at alkaline reaction, produced antibodies reacting with another unrelated protein similarly treated. Similarly, anaphylaxis could be induced by sensitizing with one coupled protein, shocking with another. Fujio considered the reaction to involve the amino groups.

7. CONJUGATION

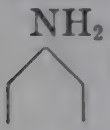
Methods (usually coupling with a diazonium compound) have been employed to attach a new specificity to a protein, and thus create an artificial conjugated antigen. In addition to doing this, treatment usually weakens the native specificity of the protein. This may in part be due to other chemical changes accompanying the conjugation, but it seems likely that it is at least partly directly due to the presence of the introduced groups.

Haurowitz, Sarafian, and Schwerin (66) found that the precipitation (by an antipseudoglobulin) of pseudoglobulin (from sheep or horse serum) was prevented by the introduction of about one hundred or more azo groups or iodine atoms per molecule. When less than fifty groups were introduced, no diminution in precipitability was observed.

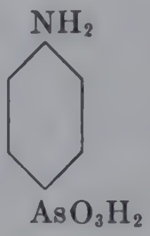
Conjugated Antigens

The specificity of antigens has been explored in minute detail by numerous workers in the present century, notably by Landsteiner (95). This has been done partly by the study of reactions with related natural proteins, but largely by the use of "conjugated antigens." By the term "conjugated antigen" is meant an antigen which has had a new specificity grafted onto it, as it were, by chemical treatment. It has been found possible to create thus new categories of specificity, so that a chemically conjugated protein will engender antibodies which will react with other unrelated proteins containing the same attached groups. The methods of doing this will be discussed briefly.

Treatment with Diazotized Amines

This is the method which was chiefly utilized by Landsteiner in his extensive studies on specificity, although he pointed out that any method leading to the attachment of new groups to the protein would be expected to have the same effect. It has proved on the whole the most flexible and useful. An aromatic amine, containing the group  NH_2 is diazotized by treatment with sodium nitrite and hydrochloric acid, and the diazonium compound $ClN{:}N$ is produced. It will couple with proteins in alkaline or even neutral solution, giving yellow, orange, or red compounds containing the diazo linkage $N{:}N.$ This results in the introduction of a new specificity, and may or may not destroy the original specificity completely, depending on the severity of the treatment. This method of introducing groups into proteins has wider possibilities than most of the other methods, because an aromatic amino group can be introduced into almost any kind of compound, making it possible then to couple it to an antigenic protein. Thus we are not restricted to the use of simple aromatic amines such as aniline and sulfanilic acid, but can make use of more complex compounds specially synthesized for the purpose, containing any chemical group we are interested in studying. An example of such a compound is the *p*-aminobenzyl ether of the specific polysaccharide of type 3 pneumococcus. With this last compound, Avery and Goebel (2) were able to immunize rabbits actively against infection with type 3 pneumococcus and to obtain antisera which precipitated the type 3 polysaccharide, agglutinated type 3 pneumococci, and protected mice specifically against type 3 infection.

According to Pauly (142–144) diazonium compounds couple with proteins through the histidine and tyrosine, two groups going into each tyrosine and two into each histidine residue. We can calculate (15) the number of entering groups per molecule of protein. Actually, Boyd and Hooker (15) and Boyd and Mover (18) found that in various proteins considerably more arsenic could be introduced by repeated treatment with diazotized arsanilic acid (below) than could be accounted for on this basis.



Introduction of too much hapten may diminish the antigenicity of the conjugate (19, 98).

Coupling through the Sulfhydryl Groups of Proteins

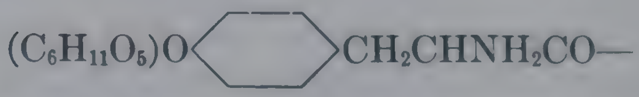
Pillemer, Ecker, and Martiensen (147) introduced groups into proteins by reducing the disulfide sulfur to sulfhydryl groups and allowing these to react with organic halogen compounds according to the following formula (X represents halogen):

$$ASH + RX \rightarrow ASR + HX$$

This method is specially applicable to keratins because of the high percentage of disulfide sulfur they contain (10–15% cystine).

Treatment with Azides

Clutton, Harington, and Mead (26) were able to introduce groups such as:



into proteins through linkage with the free amino groups, by use of the azide of *O*-β-glucoside-*N*-carbobenzyloxytyrosine. Clutton, Harington, and Yuill (27) found that this masked the specificity of the original protein entirely, and the new specificity was conditioned by the introduced groups. Gelatin was thus rendered slightly antigenic (see page 86). The latter authors (27) also succeeded in introducing in a similar way thyroxyl groups into proteins, and obtained slight reactions between the antisera and thyroglobulin. Administration of such antisera protected animals somewhat against the normal physiological effects of later doses of thyroglobulin and thyroxin.

8. Significance of Conjugation Studies

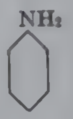
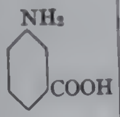
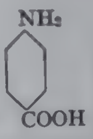
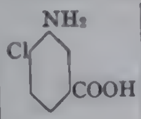
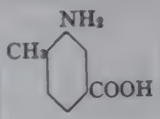
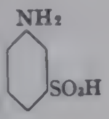
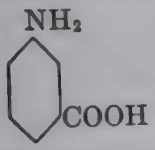
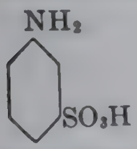
The great significance of the work of Landsteiner on artificial antigens and of its confirmation and extension by others was this: it showed that antibodies could be directed towards groups of known chemical constitution in an antigen, and *could react with these groupings by themselves.* From the point of view of the chemist, this is probably the greatest single step forward ever taken in the study of immunology, since it advanced the science at once from the stage at which it was thought that antibodies could be produced only in response to an unknown, special constitution, peculiar to proteins, to the stage at which the chemical composition of the groups determining antibody production could be studied.

Study of synthetic artificial antigens has shown that antibodies produced by them may be of several sorts. In the first place, especially if the chemical treatment has not been too drastic, the serum of an animal immunized with such a conjugated protein may contain antibodies directed towards the protein substrate, having no relation to the new specificity. It may contain antibodies directed chiefly towards the introduced groups, or these groups and the parts of the protein molecule with which they combine. Also, antibodies may occasionally be found whose combining group is directed simultaneously towards the original protein and the introduced group (64, 70, 123).

Since antisera prepared by injecting such conjugated protein antigens often contain, in addition to the antibodies against the introduced chemical group in which we are interested, antibodies against the protein carrier, we can not test for the first sort of antibodies by use of the same antigen which was injected, for we could not easily distinguish between a reaction with the introduced groups and one with the unchanged protein parts of the molecule. Therefore it is necessary to perform the test with an antigen prepared, by the same chemical treatment, from an unrelated protein. The primary reaction (see Chapter VI) will then be relatively independent of the protein.* For most purposes it will not matter if the injected antigen, and even the test antigen, consist of mixtures of proteins instead of being each a chemical entity, as the essential reaction does not concern the protein part. Landsteiner originally used horse serum for the preparation of the antigen for injection, and chicken serum for the test antigen. Purified proteins for the preparation of the antigen for injection have been found suitable, although sometimes not quite so potent (80). It has been found that purified hemocyanins and serum globulins are quite good. If it is desired to use purified proteins throughout, a hemocyanin can be used in the preparation of the antigen for injection, and a protein such as casein (which has the advantage of being more resistant to chemical treatment than, for example, egg albumin) for the preparation of the test antigen.

TABLE V

EXAMPLE OF THE SPECIFICITY OBSERVED IN ANTISERA TO ARTIFICIAL ANTIGENS OBTAINED BY COUPLING PROTEINS WITH SIMPLE AROMATIC AMINES

Antisera made with	Tested against antigens made with					
						
	0	+++	0	++++	+++	+
	0	0	0		0	++++

0 = no reaction, + positive reaction, ++++, very strong reaction.

Landsteiner and Lampl (103) coupled, among others, the two aromatic amines shown on the left of Table V to the proteins of horse serum, by diazotizing them with nitrous acid and allowing them to react with horse serum made strongly alkaline. Immune sera produced by injecting rabbits with these preparations reacted as shown in the table with antigens made by diazotizing the amines shown at the top and coupling them with the proteins of chicken serum. The degree of reaction in the different cases shows quite clearly the influence of chemical constitution on serological specificity.

---

*There is reason to think that actually at least some of the antibodies are directed, not solely against the hapten but partly against certain adjacent parts of the protein molecule (66, 78).

This experiment shows, for example, that the antibody-forming mechanism can distinguish clearly between a benzene ring bearing an acid group and one without such a group. Two different acid groups are distinguished (carboxylic and sulfonic). Furthermore, we see that the antibodies formed are specific, not only for the kind of acidic group, but also for their position, as we note that the antibodies to the compound having the carboxyl meta to the amino group (which serves as the point of attachment to the protein in the conjugated antigen) do not react to proteins coupled with the isomeric compound in which the carboxyl is in the para position. Other observations, such as a certain degree of cross reaction between carboxyl and sulfonic acid, and the relatively slight influence of groups such as chloride and methyl, could be made.

By use of such methods it has been possible to establish the limits of resolution possible by means of serological specificity. Working with various benzene ring derivatives, Landsteiner made a very large number of tests. A summary of the results will be found in Landsteiner (98).

Although in some cases the specificity seemed complete, i.e., the immune serum reacted only with the homologous antigen, more frequently cross reactions were found, although the reaction with the homologous antigen was most intense. In other words, the immunological mechanism was not able to distinguish absolutely between some of these closely related compounds. We may suppose that the antibodies, although they reflect in some way the characteristic electronic pattern of the antigen or hapten, do not reflect every detail perfectly. There will be some blurring of outlines. Or we might change the metaphor and say the antibody is a lock fitted by the hapten as a key. The lock, however, is of somewhat simpler construction than the key, so that keys for other locks may also open it, though perhaps not so readily. The cross reactions showed definite regularities, which furnish valuable information on immunological specificity, and which are best given in Landsteiner's own words (95):

"1. First of all, the nature of the acid groups is of decisive influence.

Sulfonic acid immune sera reacted markedly with several sulfonic acids, but little, if any, with carboxylic acid antigens, and immune sera to the latter only exceptionally gave distinct reactions with azoproteins containing sulfonic acid groups. The determining influence of the arsenic acid radicals was still more pronounced, as is indicated by the fact that arsanilic acid serum precipitated all of the six substances tested which contain the group AsO_3H_2, and none of the other antigens.

"2. In contrast to the acid groups, substitution of the aromatic nucleus by methyl, halogen, methoxyl and nitro groups is of less influence on the specificity. Thus in the tests presented in Table VI the immune sera act with varying intensity on almost all of the antigens possessing mono- or di-substituted benzene nuclei. The groups NO_2 and OCH_3 appear to change the specificity to a somewhat greater extent than halogen and CH_3. The radicals containing a carbonyl group constitute an exception, for the antigens prepared from acetyl-*p*-phenylenediamine and *p*-aminoacetophenone did not give any, or but weak precipitation." Hopkins and Wormall (82, 83) also found that introduction of bromine into phenyl groups did not alter the specificity significantly.

Haurowitz (65) has shown that strongly basic groups are probably just as effective as acid groups in directing specificity.

Evidently because of the strong influence of polar groups, sera to proteins coupled with phenylazo ($—N:NC_6H_5$) do not precipitate antigens containing acid groups, and conversely antisera to azoproteins containing acid groups do not react, or react weakly, with such "neutral" antigens.

Landsteiner and van der Scheer (105) found that an azoprotein prepared from the methyl ester of *p*-aminobenzoic acid reacted as a "neutral para-antigen," reacting with antisera to aniline and *p*-toluidine, and only very faintly with an antiserum for *p*-aminobenzoic acid. But, if the azoprotein was gently treated with sodium hydroxide so as to hydrolyze off the methyl group, the protein gradually lost its precipitability by immune anti-"neutral" sera, while at the same time it began to react strongly with *p*-aminobenzoic acid serum. Similarly, immune sera prepared with proteins containing the ester reacted with other proteins so treated, but not with proteins containing the plain *p*-aminobenzoic acid.

Landsteiner (95) points out that the pronounced effect of esterification of terminal carboxyl groups suggests that the terminal portions of the molecule have a particularly significant influence on the specificity. He goes on to say: "If one visualizes the protein molecule not as a straight chain but, with Svedberg, as spherical or eliptical in shape, which according to Sørensen results from the coiling up of peptide chains, this would be in agreement with the assumption that the groupings at the periphery of the molecule, oriented towards the solvent, play a prominent part in the reactions." (Compare Marrack's view, mentioned on page 256.)

TABLE VI

REACTIONS OF ARTIFICIAL ANTIGENS MADE WITH "NEUTRAL" HAPTENS (95)

Antigens from	Immune sera for				
	Aniline	*o*-Chloroaniline	*p*-Toluidine	*p*-Nitroaniline	*p*-Chloroaniline
Aniline	++±	++	+±	+	+
o-Toluidine	++	++±	+±	±	±
o-Anisidine	+	++	±	0	0
o-Nitroaniline	+	+	±	±	0
o-Chloroaniline	+±	++±	+	±	±
m-Toluidine	++	++	+±	+	+
m-Nitroaniline	+	+	+	+±	±
m-Chloroaniline	++	+±	+±	+	+
m-Bromoaniline	++	++	+±	+	+
p-Toluidine	+±	+	++	+	++
p-Anisidine	++	+	++±	+	+±
p-Nitroaniline	+	±	+±	++	++
p-Chloroaniline	++±	+	++	+±	++
p-Bromoaniline	++	+±	++	+±	++
p-Iodoaniline	+±	+	++	+±	++
3-Nitro-4-methylaniline	++	+±	++	+±	+
4-Nitro-2-methylaniline	+	±	+	+±	±
as-*m*-Xylidine	+±	++	++	+	+±
p-Xylidine	+±	++	+	+	±
Acetyl-*p*-phenylenediamine	0	0	0	0	0
p-Aminoacetophenone	±	0	+	+	0
Monomethyl-*p*-phenylenediamine	+±			+±	

Concentration of antigens 0.01%. Symbols as in Table V.
± = trace. Increasing amounts of precipitate indicated by ±, +, +±, etc.

Another important influence of acid groups is that their presence seems to define more sharply the specificity of the nucleus in which they are found, so that other substituted groups in the ring exert more influence on the specificity than they do in neutral antigens.

Landsteiner continues:

"3. Another regularity, as seen from the very specific reactions of the three isomeric aminobenzoic acids and aminocinnamic acids, is that the relative position of the acid radical to the azo-group* determined the specificity and the appearance of cross reactions."

Having studied the specificity of introduced aromatic nuclei, Landsteiner next proceeded to investigate the specificity of aliphatic chains. It was necessary to fasten these to a benzene ring in order to have a method of attaching the compound to the protein "carrier," but this feature remained the same in any series of experiments, so that the effects of varying the aliphatic portion could be studied. Table VII shows some of these results.

The compounds used were made from the dibasic fatty acids, in each case by forming a link between one of the carboxyl groups and an aromatic amino group, in which another (*p*-amino) group was developed for purposes of coupling. The part that varied was the aliphatic part, i.e., the dibasic acid. It will be seen that the immune sera against the lower acids (oxalic and succinic) were quite specific, so that lengthening or shortening the chain by only one carbon atom produced a marked difference, whereas the antisera to the higher acids (adipic and suberic) showed much stronger overlapping reactions with the neighboring members of the series.

Therefore we find, just as in organic chemistry, that, as the carbon chain becomes longer, shortening or lengthening it by one carbon makes a smaller difference. It would also seem, since the specificity of the antisera for these compounds is after all rather high, that the polar group, CONH, common to all of them, has an influence somewhat like that of the acid groups.

It was of the greatest interest to investigate the specificity of peptides (compounds of amino acids), since proteins are made up of peptide chains. Landsteiner and van der Scheer (95) carried out this investigation in analogous fashion. The peptides were combined with nitrobenzoyl chloride, and the nitro group was reduced to an amino group for coupling with proteins. It was found that the antisera to these compounds were quite specific, so that strong cross reactions were obtained only with closely related amino acids, glycine

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* The point of attachment to the protein.

TABLE VII

CONJUGATED ANTIGENS WITH ALIPHATIC SIDE CHAINS (95)

Antigens from	Immune sera						
	p-Amino-oxanilic acid	*p*-Amino-succinanilic acid	*p*-Amino-adipanilic acid	*p*-Amino-suberanilic acid	*p*-Amino-phenylacetic acid	*p*-Amino-phenylbutyric acid	*p*-Amino-phenylcaproic acid
p-Aminooxanilic acid	++	0	0	0	0	0	0
p-Aminomalonanilic acid, $n = 1$	0	0	0	0	0	0	0
p-Aminosuccinanilic acid, $n = 2$	0	++++	±	±	0	0	±
p-Aminoglutaranilic acid, $n = 3$	0	+	+	+±	0	±	±
p-Aminoadipanilic acid, $n = 4$	0	0	+++	++±	0	±	+
p-Aminopimelanilic acid, $n = 5$	0	0	+±	+++±	±	±	+
p-Aminosuberanilic acid, $n = 6$	0	0	+±	++++	±	+	+
p-Aminobenzoic acid	0	0	0	±	0	0	0
p-Aminophenylacetic acid	0	0	±	+	+±	±	±
p-Aminophenylbutyric acid	0	0	±	+±	0	+±	+
p-Aminophenylcaproic acid	0	0	±	++	0	+	+±

Concentration of antigens 0.01%. Symbols as in Table VI.
General formula of aminoanilic acids: $NH_2C_6H_4NHCO(CH_2)_nCOOH$.

and alanine, valine and leucine, and aspartic and glutamic acid. Nevertheless these related amino acids could be differentiated without difficulty.

Antisera to dipeptides precipitated most strongly the homologous antigen (Table VIII) and gave overlapping reactions with other antigens where the terminal amino acid was the same as in the immunizing antigen. This is probably again due to the prominent influence of the acid groups referred to above; it might also be thought that the terminal group would appear more prominent, so to speak, to the antibody forming mechanism, as is suggested by other experiments of a somewhat different sort. Landsteiner points out that the specificity observed here is in many ways similar to that of the enzymic splitting of dipeptides by dipeptidase. Later experiments were made with longer peptides, with similar results. With pentapeptides cross reactions due to some of the nonterminal amino acids were observed.

TABLE VIII
REACTIONS OF PEPTIDE AZOPROTEINS (111, 111a)

Immune sera	Antigens from			
	Glycylglycine	Glycyl-leucine	Leucylglycine	Leucyl-leucine
Glycylglycine	++±	0	0	0
Glycyl-leucine I	0	++±	0	±
Glycyl-leucine II	+	+++	0	+
Leucylglycine I	+	0	+++	0
Leucylglycine II	++	0	+++	±
Leucyl-leucine	0	+	0	++

Concentration of antigens 0.01%. Symbols as in Table VI.

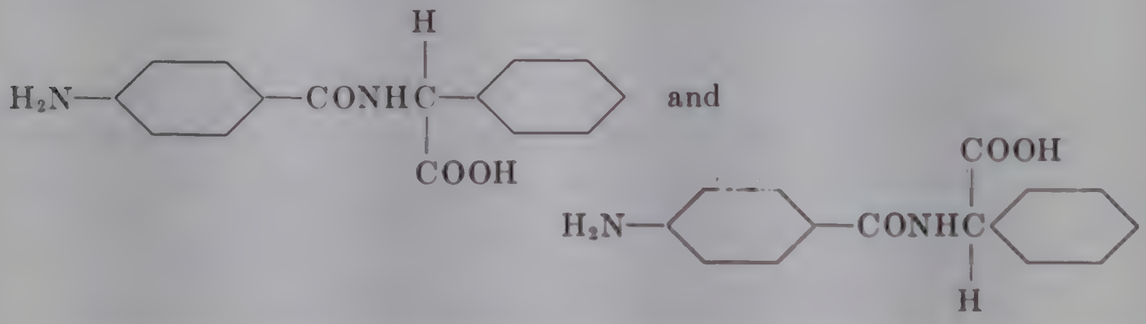
These experiments indicated that a large number of serologically different compounds could be made up from amino acids, in conformity with the large numbers of specificities found in natural proteins. They also indicate that the antibody-forming mechanism can recognize as a unit a fairly large chemical compound. Just how large a group can be responded to as a whole is not known; the maximum must be smaller than the surface of the smallest protein molecules, but possibly larger than any synthetic compound yet studied.

Nevertheless, it will be seen below that the immunological mechanism may on occasion single out parts even of chemical compounds of moderate size, so that the specificity in some cases is chiefly or solely directed towards one part of the molecule.

9. Influence of Optical Activity

The differences observed in antibodies to aromatic compounds containing the same substituents, but in different positions, indicate that the spatial arrangement of groups, as well as their chemical nature, is of importance in immunology. This is in line with what was already known of other aspects of biological chemistry, where spatial arrangement has been found important. Thus enzymes have frequently been found to be specific for one of the stereo (spatial) isomers of a substance, different isomers of optically active dyes have been found to have different staining effects, optical isomers of some of the amino acids have different tastes, and optical isomers of drugs usually exhibit different potencies.

Landsteiner and van der Scheer (95) differentiated serologically between *d*- and *l*-*p*-aminobenzoylphenylaminoacetic acids:



For a second experiment tartaric acid, which exists in three forms, dextro, levo, and meso, was used. It was coupled through one carboxyl group to an aromatic amino group, which as usual was then linked to the protein through an amino group. Table IX shows that the antisera differentiated all three of these isomers. These antisera to tartaric acid also reacted with antigens prepared with malic acid; the *d*-serum chiefly with the *d*-, the *l*- with the *l*-. It is known that the malic and tartaric acids are configurationally thus related; it would probably be possible to establish previously unknown spatial configurations by serological methods.

Later, by application of the inhibition technic (see page 441), it was possible to differentiate the isomers in a typical case of *cis–trans* isomerism (maleic and fumaric acids).

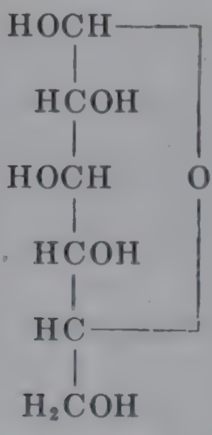
Studies by Goebel and Avery (56) carried the differentiation of steric isomers still further. These authors showed that antisera could be made against antigens containing sugar molecules linked to proteins (through the benzeneazo group), and that by the use of such sera glucose and galactose, which, as will be seen from the structural formulas, differ only in the spatial arrangement of the hydrogen and hydroxyl around the fourth carbon atom, could be clearly differentiated. Later work (4) showed that even the α and β forms of glucose, where the difference (Fig. 11) is so slight that these forms (in the case of the free sugars in solution) are spontaneously mutually interconvertible, could be differentiated. The distinction was however not so sharp as between the glucose and galactose, for the antisera also precipitated the heterologous antigens somewhat.

TABLE IX

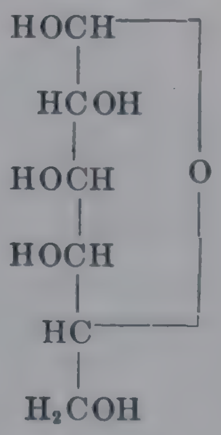
SPECIFICITY OF STEREOISOMERIC COMPOUNDS (107)

Immune sera	Antigens from					
	l-Tartaric acid		*d*-Tartaric acid		*m*-Tartaric acid	
	COOH HOCH HCOH COOH		COOH HCOH HOCH COOH		COOH HCOH HCOH COOH	
l-Tartaric acid	+++	++±	±	0	+	±
d-Tartaric acid	0	0	+++	++±	+	±
m-Tartaric acid	±	±	0	0	+++	+++

Concentration of antigens 0.05% (first column), 0.01% (second column). Symbols as in Table VI.



Glucose



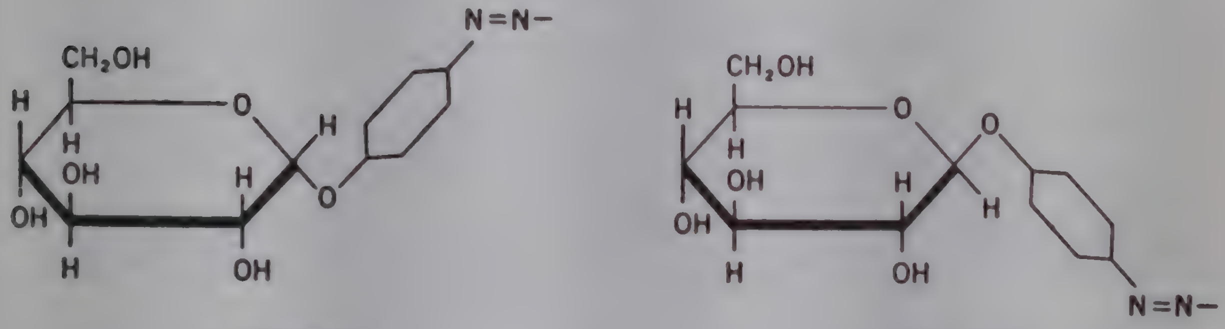
Galactose

It is apparent that in this experiment we are approaching the limits of the powers of serological reactions to differentiate stereoisomers.

It was found, as might have been expected, that the substitution of an acetyl group for the hydrogen on the sixth carbon atom modified the specificity, so that an antigen prepared from the acetyl-β-glucoside formed no precipitate with antisera to the α-glucoside, and less with the plain β-glucoside antigen than did the completely homologous acetylated β-glucoside antigen.

Goebel, Avery, and Babers (57) then proceeded to study antibodies to disaccharides, including maltose, lactose, cellobiose, and gentiobiose. Here the features determining specificity were found to be the molecular pattern of the saccharide as a whole, the spatial and chemical configuration of the terminal hexose, and the position of the linkage between the two sugars (see Table X).

Goebel (53a) found that antisera to glucose and to glucuronic acid were entirely distinct, showing no serological crossing. These compounds differ only in the groups on the sixth carbon atom. This again illustrates the strong influence of acid groups, for we have seen that the introduction of an acetyl group in the same position, though it diminished, did not abolish reactivity with the unchanged compound. It was also found that glucuronic and galacturonic acid did not cross-react serologically, similarly to the behavior of glucose and galactose.



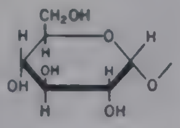
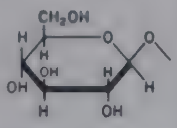
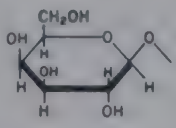
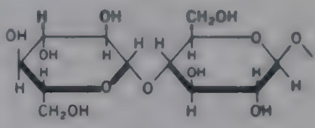
α-glucoside β-glucoside

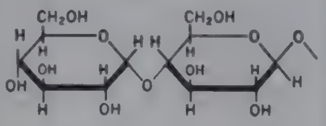
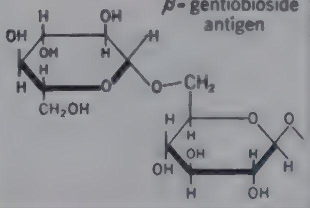
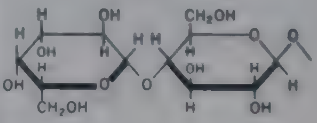
Fig. 11. Formulas showing spatial relationships of α- and β-glucosides.

It is known that the specific capsular polysaccharides of pneumococci of types 2, 3, and 8 are constituted from molecules of glucose and glucuronic acid (53a). In view of the prominent effect of acid groups, we might expect the specificity of antibodies to these polysaccharides to be directed chiefly towards the glucuronic acid. This seems to be the case, since antigens prepared from glucuronic acid are precipitated even in high dilutions by antipneumococcus horse sera of these types, while the corresponding glucose compounds ex-

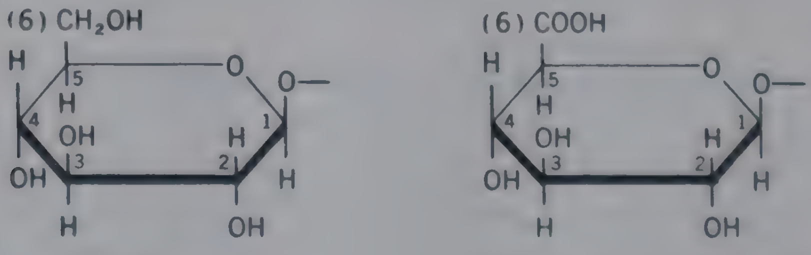
TABLE X

REACTIONS OF ANTISERA TO SYNTHETIC ANTIGENS CONTAINING VARIOUS MONO- AND DISACCHARIDES (4, 57)[a]

	Antisera to			
Carbohydrate present in test antigens	α-glucoside antigen 	β-glucoside antigen 	β-galactoside antigen 	β-cellobioside antigen 
α-glucoside	+ + ±	+		±
β-glucoside	+	+ + +	0	+ +
β-galactoside		0	+ + ±	0
β-cellobioside	0	+ +	0	+ + + ±
β-maltoside	+ +	0	0	±
β-gentiobioside	0	+ +	0	+ ±
β-lactoside	0	0	+ +	±

	Antisera to		
Carbohydrate present in test antigens	β-maltoside antigen 	β-gentiobioside antigen 	β-lactoside antigen 
α-glucoside	+ +	±	[illegible]
β-glucoside	+ +	+ +	0
β-galactoside	0	0	+
β-cellobioside	+ ±	+ +	+ +
β-maltoside	+ + + +	±	±
β-gentiobioside	+	+ + +	±
β-lactoside	±	±	+ + +

[a] Some of the structural formulas of the carbohydrate residues given in similar tables in Marrack (120) and Landsteiner (98) are wrong, and have been corrected here. Also the strength of reaction recorded has been corrected to that originally reported.



Glucose residue

Glucuronic acid residue

Fig. 12. Formulas showing spatial relationships of glucose and glucuronic acid.

hibit little or no activity (53a). It is known that type 1 pneumococcus polysaccharide contains galacturonic acid (71), so that we might expect type 1 antipneumococcus sera to precipitate antigens prepared from galacturonic acid; this also works out (58). True, it was also found that antisera for the less closely related types 3 and 8 also precipitated this antigen, but it is believed that the precipitation in this case is of a nonspecific nature.

The reactions with the glucuronic and galacturonic acids are thought by Goebel and Hotchkiss (58) possibly to be simply reactions between the acidic groups of the antigen and the basic groups of the antibody protein molecule. In experiments based on this assumption, they showed that antipneumococcus horse serum of type 3 would precipitate antigens containing other organic acid radicals (*p*-aminocarboxylic and *p*-aminosulfonic acids) quite unrelated to the uronic acids. Since the reactions occurred only with immune serum, not with normal, nor with immune serum from which antibody had been removed, we are justified in considering them as real antibody–antigen reactions.

In later work with cellobiuronic acid (a disaccharide composed of one molecule of glucuronic acid and one of glucose), which seems to be the structural unit of type 3 pneumococcal polysaccharide, Goebel (54) obtained antisera that would precipitate type 3 polysaccharide when this was combined with a heterologous protein. Antigens containing cellobiuronic acid reacted vigorously with antipneumococcus sera of types 2, 3, and 8. As would have been expected, the antiserum to antigen containing the cellobiuronic acid conferred on mice passive protection to infection with virulent pneumococci of types 2, 3, and 8 (55). This is apparently the first instance of the production of effective immunity to an actual disease by the injection of an artificial antigen containing a purely synthetic hapten. Thus the problem of specificity in the case of these organisms seems to be approaching a solution. It is worth noting that the answer has not proved as complicated as might have been predicted a few years ago. Also, the (type) specificity does not seem to depend primarily on any characteristic protein configuration, as would once have been expected.

The specificity of many other substances (called "haptens" by Landsteiner—see page 128) which can be attached to proteins and can react specifically with the antibodies produced by injecting the conjugate has been studied. The results have been on the whole similar to the above, regardless of whether the attachment was through the —N:N— group or by other methods. This work includes tests with such compounds as: $p\text{-}C_6H_5OC_6H_4NH_2$, $C_6H_5NHC_6H_4NH_2$, and $C_6H_5CH_2C_6H_4NH_2$; $NH_2C_6H_4AsO_3H_2$, $NH_2C_6H_4SbO_3H_2$, $NH_2C_6H_4SO_3H$, $NH_2C_6H_4SeO_3H$, and $NH_2C_6H_4PO_3H_2$ (40, 42); heterocyclic compounds (41, 96); thyroxine (27); pyrazolone derivatives (6, 41, 63); carcinogenic hydrocarbons (29); aminoantipyrine (95); strychnine and brucine (80); and sulfonamide compounds (53, 164, 165).

We may now offer some general considerations relative to the effect of chemical changes on serological specificity of proteins. It may be seen that quite a variety of changes destroy or diminish species specificity and produce new immunological properties even when they do not involve substitution in the aromatic rings. The exact mode of linkage to the protein does not seem to be important, and every method which has so far been attempted has been successful in producing alterations of specificity. This would seem to indicate that no particular group or part of the protein molecule is solely responsible for the specificity, but that instead it is probably determined by the general character of portions of the surface.

10. Inhibition Reaction

Although antisera against conjugated antigens containing simple chemical compounds were often found to be quite specific for the particular hapten used, and readily precipitated antigens containing the hapten coupled to another protein, nevertheless Landsteiner observed that no precipitation or other visible reaction occurred if the antisera were simply mixed directly with the hapten alone, instead of with the hapten–protein compound. This did not at the time seem surprising, since it was thought that only substances of high molecular weight, which give colloidal solutions, could take part in the precipitin reaction. (Later observations cast some doubt on this idea; see Chapter VI.)

Landsteiner, reasoning that nevertheless some reaction must occur, since the point of union in the case of the artificial antigens was obviously the hapten, took account of the fact that precipitation by immune sera is prevented or diminished when an excess of antigen is present, and deduced that the addition of an excess of the hapten might prevent the precipitation by antibody of the artificial antigen containing the hapten. Experiments proved that this actually happened, and specific inhibition of the precipitin reaction was obtained by the addition of the simple chemical compound towards which the antibody was directed, or by related compounds. The chief new result was to show that antibodies could react, not only with protein or parts of protein, but—unexpectedly—even with very simple chemical compounds, such as benzoic acid or tartaric acid.

The inhibition technic simplified the chemistry of these studies, since it made it possible to make tests on compounds without actually combining them with a protein, and in certain cases the specificity was more sharply demonstrated.

The inhibition reaction results from a union of the haptenic substance with the antibody, as was expected, and as was proved by experiments of Landsteiner (93), Marrack and Smith (119), and others (see 95). The technic is described in Chapter XI.

The results of the inhibition reactions are in general agreement with those of precipitin reactions with artificial antigens, but the reaction has furnished new information because of the ease with which it is possible to investigate numerous compounds, and because of the appearance of additional group reactions.

The reaction was seen by Landsteiner to provide a possibility of determining the nature of the specifically reacting groups in antigens of unknown composition. This possibility was realized by Wormall (170) who showed that the reactions of immune sera for iodized proteins with other iodoproteins were not inhibited by halogen compounds chosen at random, but only, of those tested, by 3,5-dihalogenated tyrosine, the effect diminishing in the order iodine, bromine, chlorine. This was taken to prove that di-iodotyrosine was the active group. The possibility that iodo- or di-iodoimidazole may also be active does not seem to have been considered.

11. Specificity of Natural Proteins

The natural proteins occur in plant and animal tissues, in the plant chiefly in the seeds. Not a great deal is known about the basis of the specificity of native proteins, but we may be confident that it is chemical in nature.

Certain workers (66, 91), as mentioned above, have obtained results from the study of conjugated proteins which suggest that in horse serum globulin, at least, tyrosine forms an essential part of the antigenic determinants. Haurowitz, Sarafian, and Schwerin (66) suggest that the determinants consist in fact of a definite arrangement of tyrosine groups, free amino groups, and perhaps other groups, on the surface of the protein molecule. These results will indicate the general bases on which we must interpret the specificity of the natural proteins, and suggest the sort of results which have been obtained. Certain general conclusions may be mentioned:

Functional Specificity

Proteins from the same species which have different functions in the body, and thus by inference (or in some instances demonstrably) in structure, usually differ widely in specificity, so that as a rule no serological cross reaction whatever is observed between pure preparations. Thus blood hemoglobin is a different protein serologically from the proteins of kidney tissue, and the serum globulins are different from the serum albumin. It has even proved possible to differentiate serologically two fractions of the serum globulins, the pseudoglobulin fraction and the euglobulin; also blood and muscle hemoglobin seem to differ. There is evidence that most organs possess special proteins or carbohydrates peculiar to them, and organ-specific antisera have been obtained. It seems quite the rule that proteins which are structurally different are also serologically different.

Species Specificity

The second general regularity we observe in the serological behavior of natural proteins is that the specificity is usually different when the proteins are from different species, even if the proteins are functionally analogous. The more closely related any two species are, apparently, the greater the serological likeness of their corresponding proteins.

There can be no doubt that these serological similarities and differences in proteins from different species are due to chemical similarities and differences in the proteins themselves. In other words, as two species diverge in the course of evolution, chemical as well as morphological differences develop, and proteins fulfilling a given function in two different species no longer have quite the same chemical composition. This is reflected in their immunological characteristics.

The most extensive investigation of species specificity was the classical work of Nuttall (134), who studied the serum proteins of a very large number of species, testing them with antisera from rabbits. Nuttall was one of the first to attempt to estimate quantitatively the differences in strength of precipitin reactions, which he did by measuring the volume of the precipitate formed. Today there are many objections which could be brought against Nuttall's work; notably his use of whole serum (a complex mixture of proteins) as antigen, instead of some one purified protein, and his lack of attention to the proportion in which antiserum and antigen are mixed, which influences the amount of precipitate. Nevertheless his work demonstrated the power of the serological method in comparative zoology, and his results have long been quoted in textbooks on evolution. Some of his data obtained with three antisera to human blood serum are shown in Table XI.

It will be seen that the intensity of the precipitation diminished in the order, anthropoid apes, old world monkeys, new world monkeys.

TABLE XI

RELATIVE AMOUNTS OF PRECIPITATE WITH THREE DIFFERENT ANTI-HUMAN RABBIT SERA AND EQUAL AMOUNTS OF BLOOD SERUM OF VARIOUS SPECIES (134)[a]

Species	Immune serum		
	1	2	3
Man	100	100	100
Chimpanzee	—	—	130[b]
Gorilla	—	—	64
Orangutan	47	80	42
Cynocephalus mormon	30	50	42
Cercopithecus petaurista	30	50	—
Ateles vellerosus	22	25	—
Cat (*Felis domesticus*)	11	—	3
Dog (*Canis familiaris*)	11	—	3

[a] Amount of precipitate obtained with human blood taken arbitrarily as 100.
[b] Loose precipitate.

The variability in the results with the different antisera is also apparent.

12. SEROLOGICAL STUDY OF ZOOLOGICAL AND BOTANICAL RELATIONSHIPS

It is clear that Nuttall's and similar results have an important bearing on the question of animal (and potentially plant) relationships. A rather large number of workers have attempted to make more precise use of serological methods in this connection. The work of Boyden (20, 20a) and his pupils is of particular interest.

Not all workers, perhaps, have taken account of the many possible sources of error in this kind of work, and this fact, coupled with the specialized terminology employed, has tended to discourage zoologists from making much use of the results of serological investigations. One important limitation is the use of practically only one species, the rabbit, as the producer of the antiserum. This has led to what Landsteiner (95) called "faulty perspective," due to the tendency of the tissues of an animal to distinguish, among antigens coming in contact with them, finer differences in the antigens of species closely related to the injected animal itself. The reason for this, which on reflection does not seem surprising, seems to be that in the injected animal (the rabbit, for instance) structural features of foreign proteins which are duplicated in some of the rabbit's own proteins do not call forth the production of antibodies, but instead the antibodies are directed towards features of the foreign antigens which are different from rabbit proteins, although structurally these may be relatively minor differences. In responding to antigens from species taxonomically distant, on the other hand, the rabbit produces antibodies concerned chiefly with main structural features, while the minor characteristics in which these distant species are different from each other tend to be ignored. Thus we find that antisera prepared by injecting the rabbit with the serum proteins of other rodents may readily distinguish relatively small taxonomic differences, whereas rabbit antisera prepared by injecting bird proteins seem to indicate a much greater serological similarity between the various species. Therefore it would be desirable, whenever possible, to use as the antibody-producing animal a species not too unrelated to the group whose relationships we wish to study, instead of using rabbits for all such experiments.

Another difficulty is due to the individual variation in antibodies produced by different animals of the same species. Apparently, not only do different species recognize different parts of antigens as the most significant, but different individuals may vary in this respect, so that antibodies of different specificity are obtained in different animals. It has also been found (79, 133, 162, 168) that the specificity of the antibodies produced by an individual animal tends to diminish with prolonged immunization, so that variation also results from differences in length of the course of immunization, and probably also in the amount of antigen injected.

Another limitation to the applicability of serological methods to taxonomy arises from the absence of any certain way of expressing quantitatively the degree of relationship shown by a serological test. The method of Nuttall, though open to objections, is still in many ways better than some alternative procedures. An application of this method, using purified proteins of known concentration, with attention to individual variations in rabbit sera and to the proportion in which reagents are mixed, was carried out by Crosby (30) on respiratory pigments of several invertebrates, with interesting results.

Probably the majority of workers since Nuttall have used the method of limiting dilution (of the antigen) to obtain a measure of the intensity of the cross reactions (20, 169, 174). These workers mostly used the "ring" (interfacial) test for their titrations. However, the titer obtained in this way is probably not an adequate index of the amount of antibody present; and since what we really want is an index of how much antibody for species A, say, is present in an antiserum for species B, the "ring" test is not really suitable. Still, such a titration does measure something, and in the hands of some workers it has proved of value.

A quantitative technic which so far has been little used was suggested by Boyd (13). It depends on determining the amount of antibody for a protein of one species which is combined with by the corresponding protein of a related species.

These various ways of estimating serological relationships quantitatively may not measure the same thing. Also, the degree of relationship of two species may differ when different proteins are used in the experiment. Thus we find that the blood proteins show considerable differences between species, while the proteins of the lens of the eye seem to be practically identical. It is not entirely clear which result ought to be given the most weight taxonomically. Nevertheless, in spite of the elements of arbitrariness which derive from individual variation in antibody production, and in spite of failure of reciprocal tests (using the animals in reverse order as antigen source and antibody producer) to indicate identical degrees of relationship, relationships based on the results of serological tests must have a certain reality and may serve as contributing evidence in appropriate cases.

Other proteins besides the serum proteins exhibit species specificity. The hemoglobins are a good example, and work done with them is on the whole consistent with the results of the extensive investigations of Reichert and Brown (151), who found that the shapes and angles of the hemoglobin crystals were characteristic for each species, suggesting, as do the serological results, that the hemoglobin of each species differs chemically from the others, at least in certain respects. Muscle proteins, fibrinogen, egg proteins, and milk proteins also show more or less species specificity. The globulins of the various organs probably should be included, and keratins (146) also seem to show some species specificity. Enzymes (e.g., pepsin, pepsinogen, and trypsin) also are different in different species (98). Wells and Osborne (see 166) made an extensive study of the specificity of plant proteins, and found that these also exhibited marked species specificity.

13. Medicolegal Tests

The species specificity of proteins makes possible the application of serological tests to forensic medicine. Striking results have been obtained (72, 134, 162). In forensic tests the particular uncertainties described above are not so serious a drawback.

We are usually interested in the differentiation of human and animal blood, but similar methods have been applied to the determination of the species origin of flesh and other parts of lower animals. The antisera are easily prepared by injecting blood serum into rabbits. Anti-human sera may react only with blood proteins of man and the higher anthropoids (see Table XI), but in many cases there may be weak cross reactions with proteins of other mammals. Such a serum can be rendered specific by "absorption." The technic of the test is given in full in Chapter XI. By such methods it is quite easy to differentiate human blood, even when dried, from that of other species. This may make it possible to prove, for example, that an accused murderer's story, that the stains found on his clothing were pig blood, is false, thus suggesting that he *may* be guilty of the crime attributed to him.

14. Organ Specificity

The term organ specificity is used in two different senses. In the first, it signifies that various organs of the body can be differentiated serologically. This is not surprising, since it would be expected that the functional differentiation of organs would carry with it chemical, and therefore serological, differences in the substances therein contained. Table XII shows results of tests carried out by Landsteiner *et al.* demonstrating organ specificity in cattle (106).

Morphologically, different parts of a cell can also sometimes be differentiated (74). See Chapter IV.

In its second usage the term "organ-specific" implies that antibodies to an organ of one species will be found to react with the substances from the corresponding organ of certain other species. In related species this is not surprising, but in some cases the cross reaction may extend rather farther taxonomically than would have been anticipated, the lens protein of fish, for example, reacting with antisera to mammalian lens (73). Somewhat similar experiments with casein have already been referred to. It has also been found possible to obtain antisera reacting with substances contained in alcoholic extracts of the brain of various species (see 167). Such antisera also react with testicle extracts (115). These reactions seem to depend on lipides extracted from the organs.

The specificity of a number of organs and secretions has been studied, including brain, kidney, suprarenal, placenta, hypophysis, stomach, intestine, lens, leucocytes, tumors, glioma, milk, and saliva. For references to the literature see Landsteiner (95).

TABLE XII

COMPLEMENT FIXATION TITERS WITH ANTISERA RESULTING FROM THE INJECTION INTO RABBITS OF SMALL AMOUNTS OF CELLULAR MATERIAL FROM CATTLE (106)

Antigen	Serum vs.			
	Trachea	Thymus	Kidney	Sperm
Trachea	80	<10	<10	0
Thymus	20	40	0	<10
Kidney	<10	0	80	<10
Sperm	0	0	0	40

In some cases an antiserum against a certain organ has been shown to damage that organ specifically (106, 131). Attempts, sometimes successful, to obtain antisera against the cell constituents of neoplastic growths were made early in the history of immunology (see 98). No such sera have yet proved to be of any clinical importance.

15. TOXINS

Toxins are antigenic poisons (28). Because of their clinical importance, they were intensively studied in the early days of immunology, and it is still customary in many books to illustrate immunological phenomena by reference to toxins and antitoxins, although we now know more about the composition of certain other antigens.

Most toxins are poisonous substances produced by bacteria. It is customary to subdivide bacterial toxins into two classes, exotoxins and endotoxins, although the general validity of such a classification can be questioned (35). The exotoxins, typified by diphtheria toxin, appear in the culture medium during growth of the bacteria and remain when the organisms are filtered or centrifuged off. The typical endotoxins appear to be intracellular constituents of the bacterial body, which are not set free during life. They produce nonspecific lesions and symptoms in experimental animals. In so far as the endotoxins are not simply a collective name for the cause of toxic reactions obtained by injecting dead bacteria or mixtures of bacterial substance, they are probably identical with the carbohydrate–lipide complexes described below under the name of Boivin antigens.

The exotoxins are generally better antigens than the endotoxins, are more toxic, are usually heat labile, and, so far as we know, are proteins. It is customary to include with the toxins also certain antigenic vegetable poisons, such as ricin, crotin and abrin, snake venom, and spider poisons, as their behavior resembles that of the bacterial toxins.

Toxins, being proteins, and antigenic, will produce antibodies if injected into an animal. There is no particular reason to think that the toxic group in a toxin is necessarily identical with the groups which act as determinants when it functions as an antigen. The antibodies to toxin will neutralize toxin, and hence are called antitoxins, but their action might be due to a blocking of the toxic groups, i.e., the latter might simply be covered up by the antitoxin molecules although these were attached to other groups. There seem to be no experimental results which would enable us to affirm or deny that this is really the case.

Although it is not generally classed as a toxin, it seems desirable to say a few words here about the chemistry of tuberculin, which derives from the tubercle bacillus, and gives reactions of a special type in tuberculous individuals (see Chapter IX). Tuberculin contains three chief colloidal components (153), protein, polysaccharide, and nucleic acid. The polysaccharide and the nucleic acid have both been isolated in pure form and studied, but neither gave any significant biological reactions, except a specific precipitin reaction between the polysaccharide and sera from animals immunized with the tubercle bacillus.

The protein portion of tuberculin is the most important because of its specific biological reactions in tuberculous animals and human beings; it is very complex. Two soluble native proteins, A and B, have been identified and distinguished by different electrophoretic mobilities. The presence of imidazole groups in these molecules is suspected. Different antibodies are formed to these different proteins. A purified tuberculin protein, known as PPD (purified protein derivative) contains more of the A protein, and is biologically the most active fraction obtained.

Neutralizing antibodies have been successfully produced for toxins from the following bacteria: *Corynebacterium diphtheriae, Clostridium tetani, Cl. chauvoei, Pseudomonas aeruginosa, Cl. botulinum, Cl. perfringens, Cl. septicum, Cl. novyi, Cl. bifermentans, Shigella dysenteriae, Streptococcus pyogenes, Staphylococcus aureus*, and for various animal toxins such as snake venoms, spider poisons, and scorpion venom, and for various plant toxins, such as ricin (from the castor bean) and abrin (from the seed of the Indian licorice, *Abrus precatorius*) (172).

In those toxins which have been studied, the toxicity seems to be an integral part of the molecule, not dependent on any prosthetic group. In several cases it has been found that the toxicity can be destroyed, without violent alteration of the molecule otherwise, by the action of formaldehyde (see below).

Snake venoms seem to contain in all cases at least two toxic proteins; some of these are enzymes. Recent work on the chemistry of animal poisons is summarized by Kellaway (90) who gives a key to the literature up to 1939.

One animal toxin, crotoxin from the rattlesnake *Crotalus terrificus*, has been crystallized by Slotta and Fraenkel-Conrat (154). It is a protein which seemed to be homogeneous and to possess a molecular weight of about 30,000 (98). Both the toxicity and the hemolytic effect seemed to depend on the lecithinase activity of the molecule. Cobra venom (98) has also been purified. Venoms seem to have considerable sulfur (four to five per cent) and five to six per cent of zinc.

Diphtheria toxin has been prepared in fairly pure form by Eaton (37) and Pappenheimer (136). Its characteristics are given in Table XIII. Petermann and Pappenheimer found that purified diphtheria toxin has a *p*H stability range of 5.6 to 10.1 in buffers 0.172 *M* in sodium chloride.

Lamanna *et al.* (92a) have recently purified and crystallized botulinus type A toxin. It seems to be a protein with a molecular weight between 1,000,000 and 2,000,000. The MLD (for mice) was found to be about 8×10^{-9} mg., in terms of nitrogen. Pillemer *et al.* have isolated and crystallized tetanus toxin. It also behaved as a protein, and the MLD for mice was about 1 or 2×10^{-8} mg. of nitrogen (145a).

16. TOXOIDS

It was early observed that toxins on storage might lose part of their toxicity while retaining their power to neutralize antitoxin. Treatment with certain chemicals had a similar effect. Ramon (150) found it possible to destroy the toxic properties completely, without appreciable loss of antigenic qualities, by incubating toxin with formaldehyde. Ramon called his product "anatoxine," but the name toxoid is more common in the English literature.

TABLE XIII

CHEMICAL COMPOSITION AND PROPERTIES OF PURIFIED DIPHTHERIA TOXIN

Composition		Properties	
C	51.47%	Specific rotation	−39°
H	6.75	Isoelectric point	4.1
N[a]	16.00	Molecular weight	72,000
S	0.75	Dissymmetry coefficient (f/f_0)	1.22
P	<0.05	Ratio of major to minor axes	4.7
Ash	1.4	Per cent N specifically precipitable	95–98
Amino nitrogen	0.98		
Tyrosine	9.5	MLD per mg.	14,000
Arginine	3.8	Mg. N per L_f unit	0.00045
Lysine	5.3		

[a] Corrected for ash.

There is no appreciable change in the amount of nitrogen in one flocculating unit when toxin is converted to toxoid. There have been some experiments indicating that the *in vivo* combining power has been somewhat affected.

Formaldehyde apparently acts directly on the toxic groups without affecting other parts of the toxin molecule which are concerned with antigenicity and combining activity. It is natural to suppose that the effect is due to combination with the free amino groups of the toxin, but Hewitt (75) pointed out that the reaction between toxin and formaldehyde to form toxoid is slow and irreversible, while that between formaldehyde and the free amino groups of proteins, polypeptides, and amino acids is rapid and reversible. The amount of formaldehyde required for toxoid formation is also less.

Goldie (59) and Pappenheimer (137) found that, although the reaction is irreversible and consequently, as mentioned above, not due merely to the formation of methylene linkages with the amino nitrogen, nevertheless toxicity was abolished by combination of other groups with the amino groups, as by treatment with ketene gas.

Toxoid is a more stable product than toxin. It does not appear to lose its antigenic value with aging, and it is more resistant to heat than toxin. Eaton (38) points out that the fact that toxin and toxoid have the same optical rotation suggests that the optically active atoms adjacent to the peptide linkages may not have been affected.

17. Specificity of Carbohydrates

Dochez and Avery (34) found in cultures of pneumococci a soluble substance precipitating specifically with antiserum of corresponding type. Zinsser and Parker (173) obtained similar precipitable products which were free from protein. Avery and Heidelberger took up the study of these products, and demonstrated that these nonprotein precipitable substances were polysaccharides (see the review by Heidelberger, 68). This discovery powerfully stimulated the chemical investigation of antigens.

It has been found that some carbohydrates can be antigenic, and that quite a number may react specifically with antisera obtained by injecting either whole organisms, or various mixtures of substances. The species specificity of carbohydrates seems often to be somewhat less than that of proteins, possibly because of the smaller number of different compounds possible in the carbohydrate series. Consequently, cross reactions with distantly related species are more common. Thus, by chemical accident (35) a rabbit antiserum for the encapsulated type B Friedländer bacillus precipitates the capsular carbohydrate of type 2 pneumococcus (and vice versa) and antisera for type 14 pneumococcus react with human erythrocytes, especially of blood group A (see Chapter IV).

The structure of five polysaccharides has been studied sufficiently to relate it to their immunological behavior (see 95, 120). These are: the specific carbohydrates of pneumococci of types 3 and 8, the polysaccharide of Friedländer's bacillus (type A), the purified polysaccharide of gum acacia, and the gum of *Penicillium luteum*.

The structure of polysaccharide S3 is probably the best worked out.

It is built up of aldobionic acid molecules united by a glucoside link. These aldobionic acid molecules are composed of one molecule of glucuronic acid and one of glucose, united by a glucoside link involving the reducing group (aldehyde) of the glucuronic acid and carbon 4 of the glucose, giving cellobiuronic acid (85). See Figure 13. These aldobionic acid molecules are joined together by a glucoside link involving the reducing group of the glucose, and leaving the carboxyl group of the glucuronic acid free (see page 204).

The polysaccharide of type 8 pneumococcus also contains cellobiuronic acid, but in addition contains glucose in some other form. The polysaccharide of type 2 pneumococcus is a weak acid, yielding glucose on hydrolysis; the nature of its uronic constituent is at present writing still unknown. Marrack and Carpenter (121) found that an antiserum against type 2 pneumococcus would react with a number of natural vegetable gums, even when these were diluted 1:10,000 or more. They tested cherry gum, acacia gum, plum gum, apricot gum, gum ghatti, mesquite gum, gum tragacanth, and flax mucilage. With all the gums except the last, the precipitate formation was inhibited by salts of glucuronic acid and glucuronides. These reactions serve well to illustrate the more limited species specificity found with carbohydrates. They raise the interesting question of the possible effect on bacterial infection of ingestion of similar carbohydrates in the diet.

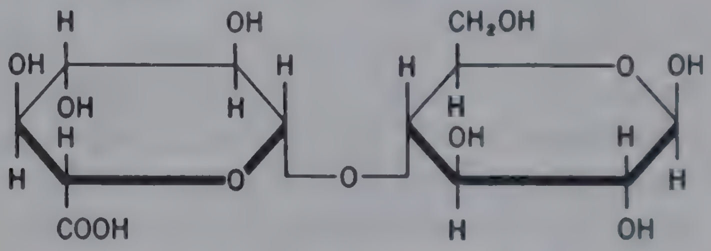


Fig. 13. Cellobiuronic acid.

The chemical properties of a number of specific carbohydrates and vegetable gums are given in Tables XVII and XVIII (pages 144 and 145) in the next chapter.

Almost all types of bacteria contain serologically active carbohydrates; the following is a partial list; references to the literature will be found in (95) (see also Chapter IV): streptococci, gonococci, meningococci, members of the *Phytomonas* and *Pasteurella* groups, *Hemophilus influenzae*, *Aerobacter aerogenes*, *Salmonella* bacilli, *Shigella dysenteriae*, bacilli of the *Brucella* group, *Proteus vulgaris*, *Ba-*

cillus anthracis, spirochetes, yeasts and fungi, *Rickettsiae*, and *Monilia*.

Reactive carbohydrates have also been found in other organisms. For example, Campbell (22) isolated from *Ascaris lumbricoides* (from hog) a nitrogen-free polysaccharide, apparently free from protein, which was antigenic.

18. Lipide Antigens

The question of the possible antigenicity of lipides has been long debated. It has not been demonstrated that any pure lipide is by itself capable of inciting antibody formation, but antibodies have been obtained by injecting lipides mixed with protein. In particular lecithin (even synthetic) and cholesterol have been found antigenic in this sense by some workers, but others have reported failure when purified lecithin preparations were used.

The *Forssman antigen* is discussed in Chapter IV.

19. Boivin Antigens (Carbohydrate–Lipide Complexes)

Boivin and Mesrobeanu (9, 10) discovered a new type of antigen. By extracting *Salmonella typhimurium* with 0.25 *N* trichloroacetic acid in the cold, they obtained a complex which consists of a non-antigenic specific polysaccharide, acetic acid, fatty acids, and phosphoric acid. This substance was not dialyzable, gave opalescent solutions, was specific, toxic, and antigenic. On hydrolysis fatty acids and the specific polysaccharide were liberated. Neither of these is toxic or antigenic. Similar results were reported by Raistrick and Topley (149), and such complexes have been isolated also from dysentery bacteria, various species of *Pasteurella*, *Pseudomonas aeruginosa*, *Bacillus anthracis*, *Eberthella typhosa*, *Proteus* X_{19}, etc. References to the literature will be found in (24). Substances probably belonging to this class have been prepared from *Vibrio comma*. Boivin considers that these substances correspond to the somatic antigen and the endotoxin of the organism. They incite the production of agglutinins of the "granular" or O type. They are very unstable.

Other workers have used, in addition to the trichloroacetic acid technic, tryptic digestion and extraction of the dry cells with diethylene glycol. Morgan (125), applying the latter technic to the Shiga bacillus, obtained a complex that engendered both heterogenetic and antibacterial antibodies, and had the properties of the Shiga endotoxin (see Chapter IV).

The Boivin antigens from a variety of Gram-negative bacteria may produce hemorrhage of tumors in animals with transplantable tumors and interrupt pregnancy at certain stages in mice and rats (88).

Morgan (126) and Morgan and Partridge (141) have been able to make artificial complexes, using the "polypeptide-like" substance isolated from Shiga, and carbohydrates such as agar and the group A substance prepared from commercial pepsin. Rabbits immunized with the agar complex produced sera specific against agar, capable of precipitating a purified substance prepared from agar up to a dilution of 1:2,000,000. Antisera against the group A complex agglutinate group A erythrocytes in dilutions of 1:3,000 to 1:12,000, but did not agglutinate group B erythrocytes appreciably more than normal sera. The "polypeptide-like" substance, which seems actually to be a simple protein plus a prosthetic group (see Chapter IV) appears to have a special power of forming antigenic complexes with polysaccharides.

20. HAPTENS

The term hapten was introduced by Landsteiner (95), and is defined by him as ". . . specific protein-free substances, although reactive in vitro, induced no, or only slight antibody response. For serologically active substances of this sort, in contradistinction to the protein antigens which possess both properties, the term hapten has been proposed. . ."

It will be seen that this definition includes most of the carbohydrates, the Forssman "antigen," and the large numbers of simple substances which have been found to react specifically with appropriate antisera. By use of the term hapten much of the confusion resulting from the two senses of the word antigen can be avoided.

Although the haptens, by definition, have little if any antigenic power, it has been found that certain simple substances, which can also serve as haptens, possess the power of producing specific skin sensitization (see Chapter VIII).

The reaction of the separate hapten may or may not be a visible one. In the case of the specific polysaccharides the reaction is usually a precipitation, but if the polysaccharide is hydrolyzed to smaller units, the precipitability eventually disappears, although the fragments may still possess specific reactive power, as shown by "inhibition tests" (prevention of precipitation with conjugated protein antigen). The technic of this reaction is described in Chapter XI. With simple chemical substances, it is generally necessary to use the inhibition reaction to demonstrate the specific reactivity, although it has been found (12, 109, 110, 140) that some azo dyes will precipitate specifically. The precipitability of these dyes might be due to peculiarities of constitution, which, like those of fatty groups, diminish solubility in water and favor the formation of colloidal solutions, although Pauling *et al.* (140) explain it by the "lattice hypothesis" (see Chapter VI), and Boyd (12) proposes an explanation called the "occlusion theory" (see Chapter VI). Some of these substances were also found to elicit anaphylactic shock, even when only a fraction of a milligram was injected. Thus two serological reactions once thought to be restricted to proteins have been found to be possible also with simple substances of known chemical constitution.

SUMMARY

The word "antigen" is used in two senses, a substance which causes an animal to produce antibodies, and a substance which reacts with an antibody. A substance may belong to both classes simultaneously, but must belong to the first to be called *antigenic.* (*1*) For a substance to be antigenic, it probably has to have a fairly large molecular weight, be foreign to the animal's own circulation (*horror autotoxicus*), and not be eliminated too rapidly. Most good antigens are proteins. Aside from this, little is known about the conditions of antigenicity. (*2*) A strong antigen may *sometimes* interfere with antibody production to a weak antigen, if they are injected together (competition of antigens). (*3*) The specificity of antigens resides in the chemical structure of their molecules, as shown by (*a*) serological differences between different purified proteins, (*b*) the cross reactions obtained with compounds structurally related (including haptens), and (*c*) alterations in the specificity of antigens resulting from chemical alteration. (*4*) The specific determinants of natural proteins seem to be combinations and arrangements of amino acids. Artificial determinants (haptens) can be introduced. The average size of a specific group may be of the order of 600–1000 (molecular weight), but occasionally very simple artificial chemical groupings may act as specific determinants. (*5*) The number of reactive groups (valence) of an antigen molecule must be at least as great as the number of antibody molecules which can combine with it, and may be more. Therefore the valence of such antigens as egg albumin and serum albumin must be at least five or six. Larger proteins have higher valences. The combining groups must be on the surface of the molecule. Studies on artificial protein–hapten complexes suggest valences of about the same order of magnitude. (*6*) Almost any decided chemical change in a protein alters its specificity. Various workers have investigated denaturation; oxidation; reduction; digestion; the effect of acid and alkali; deamination; substitution reactions employing formaldehyde; esterification; acylation; halogenation; nitration; diazotization; treatment with mustard gas, phenyl isocyanate, β-naphthoquinone sulfonate; and conjugation with various natural and artificial compounds (haptens). (*7*) Conjugation may be accomplished through the azo linkage by using diazotized amines, or through the sulfhydryl groups of proteins, or by treatment with azides. (*8*) The great significance of the work of Landsteiner on conjugated antigens was that it showed that antibodies could be directed towards groups of known chemical constitution in an antigen, and *could react with these antigens by themselves.* Specificity was found to be very good, but to have some limits. That is, when the haptens were too similar chemically they were not well distinguished. Haptens containing polar groups, especially strong acidic or strong basic groups, were more specific than "neutral" haptens. The position in the hapten of various substituents was also important. Different peptides showed different specificities, the terminal amino acids having more influence than the others. (*9*) Differences in spatial configuration (and therefore optical activity) of an antigen or a part of it may be detected serologically. Differentiating the α and β forms of glucose seems to be approaching the limits of the powers of serological resolution. In some cases cross reactions were obtained with natural polysaccharides and rather simple substances. Other substances, such as various alkaloids and sulfa drugs, could often be differentiated serologically. It would seem that no particular group or part of the protein molecule is solely responsible for the specificity of a given molecule. (*10*) Simple haptens which may fail to give a visible reaction with antibodies directed towards them may nevertheless combine, as shown by their power to prevent reaction with protein–hapten conjugates added to the mixture later (inhibition reaction). (*11*) Not much is known about the structural basis of the specificity of natural proteins; tyrosine has been suggested as an essential feature in some cases, but is clearly not necessary in all cases. Proteins, even from the same species, which have different functions in the body may differ widely in specificity. Functionally analogous proteins from different species usually differ in specificity, although cross reactions may be observed. (*12*) Serological methods can be and have been used to study zoological and botanical relationships (Nuttall, Boyden). These investigations have provided interesting information, conforming on the whole to what is known of taxonomic relationships, but many pitfalls are encountered. *(13)* The species specificity of proteins makes it possible to apply serological tests to forensic medicine, and such methods have been considerably used to differentiate human and animal bloods. (*14*) Organ specificity is a term used to mean that various organs of the body can be differentiated serologically, and that analogous organs of related species may cross-react with antibodies. (*15*) Toxins are antigenic poisons. We consider chiefly those from bacteria, which have been customarily classified into exotoxins, which appear in the environment or culture medium of the bacterium, and the endotoxins, which are not set free during the life of the bacterium. The endotoxins are generally less toxic, and may be certain constituents of the bacterial cell, perhaps in some cases the carbohydrate–lipide complexes isolated by Boivin and others. Tuberculin, a protein when purified, is not strictly a toxin. It may contain imidazole groups. Neutralizing antibodies (antitoxins) have been successfully produced for a variety of toxins. Diphtheria toxin has been prepared in fairly pure form. It is a protein of about 70,000 molecular weight. (*16*) Toxins may often be detoxified (usually by formaldehyde) without losing too much of their antigenicity. Toxoids may sometimes find successful application in immunization to disease, as with diphtheria and tetanus. (*17*) Carbohydrates exhibit serological specificity, and some are somewhat antigenic. Pneumococci are classified into types on the basis of reactions produced by antibodies to the various capsular polysaccharides. The chemical structure of some of these, particularly that from pneumococcus type 3, is known to some extent. Practically all sorts of bacteria contain serologically active carbohydrates. Cross reactions are perhaps more common than with proteins. (*18*) It has not been demonstrated that any pure lipide is by itself capable of inciting antibody formation, but some may when mixed with protein. (*19*) Boivin antigens (lipide–carbohydrate complexes) have been extracted from microorganisms by Boivin, Morgan, and other workers. A polypeptide or protein-like component may be present. Such antigens may be quite potent. (*20*) Haptens were defined by Landsteiner as specific protein-free parts of the antigen, reactive *in vitro*, but not antigenic alone or only slightly antigenic. A few synthetic haptens have been found to precipitate directly with antibody.

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CHAPTER IV

CELL ANTIGENS

Hitherto we have spoken chiefly of antibodies to individual antigenic substances. In clinical medicine, however, what interests us is usually resistance to the living agent of a disease, which is often a microorganism having a chemically complex structure. Also, many theoretical studies are based on antibodies to complex cells such as erythrocytes. Human isoagglutinins for the red cells of human blood are of the greatest importance in transfusion (see Chapter V), and lysins for erythrocytes have been very useful in laboratory work because of the ease with which hemolysis is observed, and the ease of preparing homogeneous cell suspensions. Bacterial agglutinins have found wide application because they aid in the identification of bacteria when known immune serum is allowed to act on the bacteria, as in the Gruber-Widal test for typhoid. They have made possible the subdivision of various bacterial species into serological strains (see below).

It will readily be understood that such antibodies to cells are not directed towards the cell as a unit. The cell is a complex mosaic of proteins, lipides, and carbohydrates, and it is conceivable that any of these, after being taken up by the reticulo-endothelial system, might function as an antigen, for, although it was formerly assumed that of the constituents of the cell only the proteins were antigenically active, it is now known that in addition the carbohydrates are important, lipides play some role, and carbohydrate–lipide complexes may be good antigens (Chapter III). The antigenicity of the cell as a whole often seems to be greater than that of any of its components, at least after the rather drastic chemical treatment these have usually undergone during the process of isolation.

Cell antigens possess certain distinguishing characteristics which justify us in treating them in a separate chapter. For example, cells of closely related species, and even of individuals of the same species (blood groups), may differ strikingly in antigenic constitution, and substances reacting similarly may occur in distantly related animals, and even plants (heterogenetic antigens). Landsteiner (59) suggested that these features, together with observations on the chemical nature of the antigens, indicate that two systems of species specificity exist in the animal kingdom, the specificity of proteins and that of haptens.

Precipitins for foreign proteins seldom occur in normal serum but agglutinins and lysins for cells are found fairly often. In view of what was said above about "normal antibodies," this might be taken as an indication that antibodies to cell antigens display in general a lower degree of specificity.

TABLE XIV

LIMITING TITERS OBTAINED WHEN THREE DIFFERENT IMMUNE AGGLUTINATING SERA WERE TESTED AGAINST BLOOD CORPUSCLES OF FOUR SPECIES (63)

Blood corpuscles of	Immune serum against		
	Human blood	Chimpanzee blood	Rhesus blood
Man	400	160	60
Chimpanzee	200	640	40
Baboon	80	40	800
Macacus rhesus	80	40	800

1. DIFFERENTIATION OF CLOSELY RELATED SPECIES

Agglutinating sera prepared by injecting blood corpuscles of one species will also, analogously to precipitins, react with the corpuscles of related species. We have already presented some of the results of Nuttall with precipitins for human and primate blood; in Table XIV will be found analogous reactions of immune agglutinating sera prepared with human and monkey blood. The numbers represent the highest active dilutions of the serum (limiting titers).

A better differentiation between blood cells of closely allied species can be achieved by absorption experiments, that is, by treating the serum with enough of the heterologous antigen to remove all activity for it; there will then be found more specific antibodies remaining for the homologous antigen (64). This method also finds application in experiments on bacteria. A typical (hypothetical) example of this method is shown in Table XV, which illustrates how three closely related species could be differentiated by the absorption technic.

Not only different species, but different individuals of the same species, can sometimes be differentiated by such agglutination reactions (blood groups), something which has never been accomplished with precipitins. These blood differences found within the same species will be discussed in Chapter V.

2. The Forssman Hapten

The recognition of the presence of serologically related substances in the cells of animals which are widely separated in the zoological system is due to Forssman (31), who found that injection of ground-up guinea pig organs into rabbits produced lysins of high titer for sheep erythrocytes. The Forssman hapten is found widely distributed in the animal, and even the plant, kingdom, but, as will be seen in Table XVI, its distribution is not wholly random. It is possible that more extensive investigations would reveal still more order in its taxonomic distribution.

Table XV

Ideal Example of Differentiation of Bacterial Species by Agglutinin Absorption (Castellani Experiment) (19)

Immune serum for	Absorbed with	Titer when tested against		
		"A"	"B"	"C"
Bacillus "A"	(Unabsorbed)	4000	2000	2000
	A	0	0	0
	B	2000	0	2000
	C	2000	2000	0
Bacillus "B"	(Unabsorbed)	2000	4000	2000
	A	0	2000	2000
	B	0	0	0
	C	2000	2000	0
Bacillus "C"	(Unabsorbed)	2000	2000	4000
	A	0	2000	2000
	B	2000	0	2000
	C	0	0	0

Hybrids may sometimes develop antigens not found in either parent. Irwin (44–46) and associates have studied this phenomenon in hybrids of dove and pigeon species.

The "Forssman antigen" is probably not a definite chemical en-

TABLE XVI

OCCURRENCE OF FORSSMAN ANTIGENS IN VARIOUS GROUPS OF ANIMALS AND PLANTS (14, 97, 104)

Present	Absent	Present	Absent
PLANTS		*Whales* Whales *Perissodactyla* Horse	
Spinach (?)	Oats Beans Rice Mushrooms	*Artiodactyla* Camel Goat Sheep	Cattle Deer Pig Roe
MICROORGANISMS			
Pasteurella cuniculicida[a], certain salmonella *Shigella dysenteriae*[c] *Pneumococcus* *Bacillus anthracis*	*Vibrio comma* *Escherichia coli* *Eberthella typhosa* *Salmonella enteritidis* *Mycobacterium tuberculosis* *Staphylococcus* Yeast *Shigella dysenteriae*[d]	*Rodents* Guinea pig Hamster Mouse (?) *Procyonidae* *Cercoleptes caudivolvulus* *Procyon lotor* (Raccoon) *Canidae* Dog Fox *Lycaon pictus*	Rabbit Rat
ANIMALS			
Worms *Trichinella spiralis* *Crustacea* Lobster (?) *Orthoptera* *Mollusca* *Fish* Carp[b] Eel[b] Pike[b] Tench[b] *Amphibia* Toad *Reptiles* Turtle *Birds* Chicken Ostrich Turkey buzzard	Meal worm Tapeworm Cockroach Various shell fish Codfish Herring Frog Cuckoo Crossbill Goose Owl Pigeon Sparrow hawk Wagtail	Wolf *Felidae* Cat Lion Ocelot Puma Tiger *Primates* Lemurs (1 species) *Nyctipithecus trivirgatus* Man (group A)[e]	*Cebus* (2 species) *Cercocebus albigena* *Cercocebus fuliginosus* Chimpanzee Gibbon *Hapale jacchus* *Macacus rhesus* Man *Papio hamadryas* *Presbytis maurus* *Pygathrix cristata*

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[a] Certain strains

[b] Germ cells only

[c] R type.

[d] S cultures.

[e] Using the term "Forssman antigen" in the broader sense.

tity, but a serological conception, a collective term covering substances which, injected into rabbits, produce sheep hemolysins. The original Forssman antigen was a concept somewhat more narrow; the "heterogenetic" (or "heterophil") antisera produced by it had certain properties, i.e., they contained no lysin for cow cells, no or almost no agglutinin for sheep red cells, and their antibodies were absorbable by tissues containing the Forssman antigen (horse and guinea pig kidney).

Almost every rabbit will respond promptly to these original Forssman antigens (F antigens). They are resistant to boiling and are alcohol soluble. They have been studied chemically by Landsteiner and Levene (61, 62) and by Brunius (17). They usually occur in the organs and not in the erythrocytes, but in some cases occur only in the erythrocytes (sheep and goat) and may occur in both (chicken).

Several authors have used the term F antigen for all types of substances producing sheep hemolysins, except material from sheep or closely related species. The antibodies obtained are not identical *inter se* as the original F antibodies were supposed to be. Some data on the F antigen are based not on immunization experiments but on absorption tests. In certain cases it might be doubted (Schiff) if the antibody observed was a real immune F antibody.

Landsteiner and Levene found the purified Forssman substance to be soluble in water, dilute alkali, or pyridine, but not soluble, or barely so, in most organic solvents. Analyses gave: carbon, 55–58%, hydrogen, about 9%, and nitrogen, 2–3%. Reducing sugar was obtained on hydrolysis. Brunius found carbon, 59.5–63.8% and nitrogen, 2.0–2.3%. On hydrolysis hexosamine was found. Chase and Landsteiner (21) state that another carbohydrate, probably a hexose, is also present.

In addition to the serological cross reactions between distantly related species due to the Forssman antigen, there are a number of other cross reactions thought to be due to a common antigen, or some common structure in carbohydrates or proteins of certain species. The following groups having such serological properties in common have been found (references in 14): *Macacus rhesus* — pig; man — Shiga bacillus; horse — rat; *Pasteurella cuniculicida* — birds; man — dog — pig; man — rat — rabbit — guinea pig; cat — horse; pig — horse — sheep; man (group A) — cattle; man — *Macacus rhesus;* rabbit — pig; man — pneumococcus type 14. The chemical nature of most of these haptens is still unknown.

The Forssman antigen is not a "complete" antigen, because injected alone it does not produce antibodies. To obtain these it is necessary to mix it with something else. It is customary to use pig serum for this purpose, though other proteins, even serum from the animal species being immunized, will do. It has also been found that the Forssman antigen, if it has not been purified too much, will produce antibodies when injected adsorbed on kaolin or charcoal (see Chapter II). When protein is used in such experiments it is called a "Schlepper;" Landsteiner supposes that its efficacy is due to a loose combination with the Forssman antigen (hapten) (60).

Furth and Kabat (33) found materials of high molecular weight in all normal and neoplastic tissues. The Forssman and Wassermann activity seemed to be carried by these substances.

3. Localization of Cell Antigens

Serological methods have been applied to the study of the localization of different antigens in the cell. When antibody directed against a particular chemical constituent of a microorganism agglutinates suspensions of these organisms, it is evidently necessary to suppose that this particular antigen is located, at least in part, on or near the surface. Some of the best examples will be found in studies on bacteria, some of which are about to be discussed, but an interesting example is also provided by the experiments of Henle *et al.* (40) on mammalian spermatozoa. These workers found one antigen (heat-stable) common to the heads of the spermatozoa and to the tails. This antigen was species specific. Both heads and tails contained (heat-labile) antigens peculiar to themselves. By use of the different antisera two different types of agglutination, head–head and tail–tail, could be demonstrated, so these antigens are evidently on the surface. In the heads, an interior antigen was demonstrated by breaking up the spermatozoa before injection.

4. Bacterial Types

Bacteria as a group are probably a heterogeneous collection of more or less unrelated forms (26), classified together because of their being roughly the same size. Naturally, therefore, the antigens contained in them may vary widely.

If serological methods are applied to the differentiation of different bacteria, it is found that, while in general the results parallel the accepted classification of these organisms, species which on the basis of morphological and cultural characteristics are classified together may exhibit sharp differences in serological behavior, and the species may thus be subdivided into types. Such differences have been found in many sorts of bacteria, and in some cases the number of types is quite large. They may or may not be correlated with some detectable difference in cultural or pathogenic behavior.

Bacteria have long been classified into Gram-positive (retentive of gentian violet after mordanting with iodine and washing with alcohol) and Gram-negative (not retentive of gentian violet under such treatment) forms. Dubos (26) believes that the Gram-positive character may reside in a protein–ribonucleate complex which exists at the bacterial cell surface.

Capsule Formation

Many investigators are inclined to believe that bacterial capsule formation corresponds to the accumulation around the cell of an excreted product, which, on account of its viscosity and certain properties of the environment does not diffuse readily into the surrounding medium (26).

Pneumococcus Types

The existence of *pneumococcus* types was definitely shown by Neufeld and Händel (84), who found that immune sera protecting mice against certain strains of pneumococci were not effective against other strains, and that these differences were correlated with differences in agglutination reactions. The study of pneumococci has resulted in their classification into a large number of serological types (22, 23, 52, 109). These types differ because of serological differences in the polysaccharides of the capsule. Many of these polysaccharides have been studied and found to be chemically different (references in 16, 47, 70). Some of these results, together with figures for other serologically reactive polysaccharides, are shown in Tables XVII and XVIII, taken mainly from Marrack (70) and Brown (16).

It has already been mentioned that the capsular polysaccharides of pneumococci of types 1, 2, 3 and 8 are made up of molecules of glucose and glucuronic acid (34). The structures of the polysaccharides of types 3 and 8 have been discussed in Chapter III. We may recall that the type 3 polysaccharide has been shown to be made up of units consisting of cellobiuronic acid (42). The linkage between these units in the intact carbohydrate has now been shown to

TABLE XVII

CHEMICAL COMPOSITION OF SOME PNEUMOCOCCUS CAPSULAR POLYSACCHARIDES

Source of material	$[\alpha]_D$	Acid equivalent	Per cent of						Components identified
			C	H	N	P	Reducing sugar	Acetyl	
Pn 1	+265 – +277	576	42.55	6.58	4.85	0–0.25	32	7.1–10	Galacturonic acid, amino sugar
Pn 1	+255.6				4.84	0.19		6.83	
Pn 2	+54 – +58	970 (600)	(45.8)	(6.4)	0.14– 0.73	0.1– 3.70	86– 95	0.4–3.8	Glucose, aldobionic acid, uronic anhydride
Pn 2	+56.8				0.26	0.06		1.52	
Pn 3	−36.0	330– 350	(42.6)	(5.6)	0.08	0–0.01	84	0.5–0.9	Glucose, glucuronic acid
Pn (species-specific)	+42	1050			6.1			Present	Amino sugar, H_3PO_4

Pn = pneumococcus. For references to the literature, see (14, 16, 47, 70). Parentheses indicate values which are somewhat uncertain.

TABLE XVIII

CHEMICAL COMPOSITION OF SOME OTHER SEROLOGICALLY ACTIVE POLYSACCHARIDES

Source of material	$(\alpha)_D$	Acid equivalent	Per cent of						Components identified
			C	H	N	P	Reducing sugar	Acetyl	
Fr. A	−100	430	43.95	6.0	0		65		Glucose, aldobionic acid, disaccharide
Fr. B	+100	680	44.6	6.1	0		70		Glucose, aldobionic acid
Fr. C	+100	680			0		70		Glucose, aldobionic acid
Anthrax bacillus	+62				4.1		59	Present	Glucose, glucosamine
Gum acacia	−10.7	800			0.8		68		Galactose, glucuronic acid
T. B. Fraction C	+85–95	ca. 7000			0.1	0.2	87		*d*-Arabinose, *d*-mannose
T. B. Fraction B	+10–41	ca. 2000			0.6–0.8	0.2–1.0	61		*d*-Arabinose
B. C. G.	+77.4	1244			0.8	0.9	77		*d*-Mannose, *d*-arabinose, inositol
B. dys. (Shiga)	+98	9000	41.9	6.35	1.61		97	Present	Hexosamine
A subst. (horse)	+10.4		44.56	6.91	7.08		57.4	9.43	Galactose, amino sugar
A substance (pig pepsin)	+16		46.88	6.62	6.16		70.7	9.95	Galactose, glucosamine*
A substance (Difco peptone)	+11.5		46.68	6.53	5.85		73.0	9.56	
Human saliva (A)	−11.5				5.7		61	10.3	Glucosamine

Fr. = Friedländer bacillus, B. dys. = Shiga bacillus, T. B. = tubercle bacillus, B. C. G. = modified tubercle bacillus (B. C. G.). A substance = blood-group-specific A substance; the A substances contained from 0.08–1.96% sulfur. For references to the literature, see (14, 16, 47, 70, and E. A. Kabat, J. Exptl. Med., **85**, 685 (1947)).

* And small amounts of a number of amino acids (15).

be through the reducing groups of one to the third carbon atom of the glucuronic acid residue of the other (89). If we assume that this linkage has the β- configuration, as there is reason to assume (89), the constitution of the structural unit of this important cell antigen becomes completely known, and according to Reeves and Goebel (89), it is:

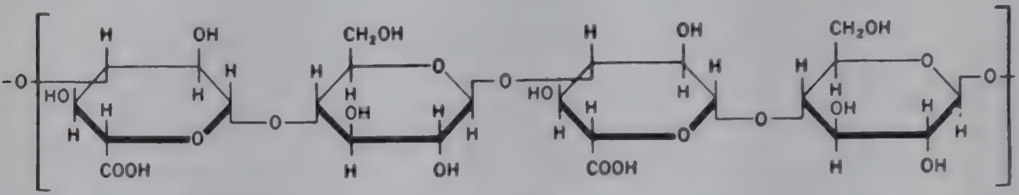

Fig. 14. The structural unit of the type 3 pneumococcus polysaccharide.

The minimal molecular weights of the capsular polysaccharides of types 3 and 8 have been calculated to be 62,000 and 140,000, respectively (38).

The specific carbohydrate of type 1 pneumococcus differs from S2 and S3 in containing about 5% nitrogen as a part of the molecule. Half of this nitrogen can be liberated by treatment with nitrous acid, and is probably present in the form of an amino sugar. The basic unit of the molecule seems to be a trisaccharide containing two molecules of uronic acid (possibly both galacturonic acid) and two atoms of nitrogen.

As the polysaccharide S1 was first isolated, it contained no acetyl groups, and would not remove all protective antibodies from type 1 antiserum. It was later found that if it were prepared without the use of alkali it contained 5.9–6.9% acetyl, was antigenic, and did remove all the protective antibodies. Treatment of this product with alkali split off acetyl groups and gave a carbohydrate identical with that originally isolated.

The molecular weights of these carbohydrates have proved hard to establish. Those first isolated probably had molecular weights somewhere in the range 1000–10,000. But Heidelberger, Kendall, and Scherp (39) found that specific polysaccharides S1, S2, and S3 prepared by milder methods, avoiding high temperatures, strong acid, or alkali, were much more viscous, and probably therefore of higher molecular weight. The new preparations also gave more precipitate with specific rabbit antisera than did the old.

So far as is known, all types of pneumococci contain the same proteins (25). The differences between the various types of pneumococci seem to reside solely in these capsular differences, and it has been found possible to convert one type into the other under the influence of substances contained in the cocci (1, 24, 32). The isolated polysaccharides alone did not seem able to bring about this transformation, although the dead bacteria could.

Avery *et al.* (9) have shown pretty conclusively that the substance which brings about the transformation of rough (nonencapsulated) pneumococci into smooth (encapsulated) type 3 pneumococci is a highly polymerized, viscous form of desoxyribonucleic acid. Similar substances would induce the transformation to other types (66, 67). This discovery does much to clear up the mystery hitherto surrounding the mechanism of the transformation, and has far-reaching implications for all future thinking about development in general.

An antigenic relationship which has been observed between the capsular polysaccharides of pneumococcus type 14 and human erythrocytes is of theoretical and practical interest. Horse pneumococcus type 14 antisera contain agglutinins for human erythrocytes of all four major blood groups in high titers (30). Antipneumococcal horse sera of other types do not exhibit this phenomenon. These agglutinins are evidently antibodies directed against the capsular-polysaccharides of the organism, for they can be removed by treatment of the serum with type 14 polysaccharide (112) as well as with the whole organism (30). They evidently cause the agglutination of human erythrocytes by combining with carbohydrates contained in these cells. Beeson and Goebel (10) succeeded in removing most, but not all, of the type 14 antibody and of the hemagglutinins by treatment with the purified group A substance, which has been shown to be similar chemically to the type 14 polysaccharide (35). It is now believed that these hemagglutinins were responsible for some of the fatal reactions which sometimes followed the use of this serum (30). Rabbit antipneumococcus sera of type 14 (but also of other types) often, but not always, contain agglutinins for human erythrocytes of groups A and AB. In these animals, evidently, the antibodies produced show an even clearer relationship to the A substance, but the relationship is not restricted to type 14 pneumococcus, as it is with the horse. In the horse, as a matter of fact, the relationship is closer to bloods of group O (and subgroup A_2) than to group A (subgroup A_1) (65).

A cross reaction between type 3 and type 8 (with antisera from the horse) has been investigated quantitatively by Heidelberger *et al.* (38).

Dubos (26) states that it is simply a chemical accident that antisera to the encapsulated type B Friedländer bacillus precipitate the capsular polysaccharide of type 2 pneumococcus, and vice versa. The two organisms, one a Gram-positive coccus, the other a Gram-negative bacillus, are phylogenetically entirely unrelated. The capsular polysaccharides simply happen to be chemically similar.

An antigenic relationship has been observed between type 6 pneumococcus and *Hemophilus influenzae* of type B (83, 114).

The procedures used in typing pneumococci are discussed in Chapter XI.

Immunologically reactive polysaccharides have been found in many other bacteria, but their chemical nature is not so well understood, and their value in classification has not always proved so great as it was in the pneumococcus. A partial list of bacterial and other specific polysaccharides is given by Landsteiner (60). It includes, in addition to the microorganisms specifically treated at some length in later sections of this chapter, *Vibrio cholera*, *Meningococcus*, *Bacterium proteus*, *B. lactis*, *Bacterium aerogenes*, *Klebsiella rhinoscleromatis*, *Bacillus anthracis*, the *Phytomonas* and *Pasteurella* groups, *H. influenzae*, *Malleomyces mallei*, *Fusobacterium*, *Corynebacterium diphtheriae*, *Clostridium welchii*, spirochetes, *Asterococcus*, *Rickettsiae*, yeasts and fungi, and helminths and other worms.

Hemolytic Streptococci

The *hemolytic streptococci* have been studied by a number of authors. The first main contributor to the problem of their classification by agglutination reactions was Griffith (37), who differentiated 27 types of pathogenic hemolytic streptococci isolated from human infections.

Lancefield (54, 55) has classified the streptococci by means of the precipitin reaction, using extracts from the organisms. She has differentiated at least three antigenic components in the extracts. One, called the M substance, is an acid-soluble protein, which is different in different types; another, called the C substance, is a complex carbohydrate, different in different groups of types. Lancefield has separated a number of groups of streptococci, designated by letters. Group A, nearly all isolated from human infections, all share the same C substance, and are differentiated into types by different M substances. A type-specific T antigen, of unknown nature, is also present (56). The glossy variants are often deficient in M. Some strains contain M but not T, some have distinct T antigens, while some have been found with related T but distinct M antigens (57, 58). Group B, mostly from cattle, possess a C substance, different from that of group A, or other groups, in common. Group B is divisible into antigenic types, not by acid-soluble proteins, as in group A, but by other complex carbohydrates. In addition to the above, a less specific nucleoprotein antigen is present (81).

The M substance is a nucleoprotein, containing nucleic acid of the ribose type, held loosely in combination in a salt-like compound. It seems to have a molecular weight of about 41,000, and is asymmetrical, having an axial ratio of about 25 to 1 (85, 115). Zittle and Mudd suggest that the M protein is a highly specialized part of the cell membrane, perhaps having a protective role (for the microorganism) similar to that assigned to the capsule of the pneumococcus, but not otherwise necessary for the normal function of the streptococcus.

This grouping of streptococci has been carried down to the letter K, and it is highly unlikely that this comes anywhere near exhausting the types which actually exist.

Staphylococci

Two types (A and B) of *staphylococci* have been characterized by polysaccharides (49). Additional types, including a clearly defined type C, have been found by Thompson and Khorozo (105). Type-specific proteins, distinct from the polysaccharides, have been found in types A, B, and C by Verwey (108).

Diphtheria Bacilli

The toxin of *C. diphtheriae* was discussed in Chapter III, methods of producing it will be mentioned in Chapter XI. Three types, *mitis* (mild), *intermedius*, and *gravis* (severe) of this bacillus (and the disease it produces) are recognized, although McLeod (69) found a few strains which could not be classified satisfactorily under any of these types.

Specific "lipoid antigens" have been demonstrated by Hoyle (43).

Acid-Fast Bacteria

The *acid-fast bacteria (mycobacteria)* can not at present be differentiated as well on the basis of cell antigens as by culturing methods. It has been found possible to distinguish four serological types by agglutination, absorption of agglutinins, and complement fixation (see 107). These types are: mammalian, avian, cold-blooded, and saprophytic. The human and bovine types are not distinguishable by this means.

The chemical structure of the acid-fast bacilli has been extensively studied by Anderson and his colleagues (3–6). A phosphatide, a polysaccharide, and various lipoids and waxes were obtained. Dubos (26) points out that there is no good evidence that the tubercle bacillus is surrounded by a waxy capsule, although this has long been believed.

The phosphatide has been found by Sabin and her colleagues (90) to be capable, on repeated intra-abdominal injection into rabbits, of giving rise to massive tuberculous tissue. One of the active constituents appeared to be a fatty acid named phthioic acid, the structure of which is not yet fully known.

The acid-fast bacteria contain a large proportion of ether-soluble constituents so firmly bound to the cellular structure that they can not be removed by extraction with neutral solvents. They are called the "firmly bound lipoids;" they represent about 12% of the human tubercle bacillus. On saponification these lipoids give fatty acids, which are almost wholly optically active hydroxy acids of high molecular weight, and specific polysaccharides. The hydroxy acid is different in each type of bacillus.

The active principle of tuberculin has been found to be a protein by Seibert (92), who has obtained it in crystalline form. Tuberculin from the bovine bacillus proved to have a molecular weight of about 10,000; tuberculin from human tubercle bacilli had a molecular weight of about 32,000 (93). The tuberculin polysaccharide was found to have a molecular weight of about 9,000.

Seibert *et al.* (93) found some indication that the protein still associated with carbohydrate is somewhat more toxic for tuberculous animals than is pure protein alone. The carbohydrate itself was not toxic. Maschmann (71) reported the preparation of two fractions from tuberculin; one, a protein, was very active in skin tests, the other, not attacked by proteolytic enzymes, was not very reactive in the skin, but quite toxic for tuberculous animals. Maschmann did not believe it to be a carbohydrate. Tennent and Watson (104a) isolated genus-specific carbohydrates from tubercle and leprosy bacilli.

Meningococcus

The *meningococcus* has been classified on the basis of agglutination eactions into various numbers of types. Topley and Wilson (107) suppose, on the basis of Gordon's work, that the types are determined by the presence of varying proportions of four antigens, designated as A, B, C, and D. See Figure 15.

Kabat *et al.* (51) prepared the type-specific polysaccharide of type I meningococcus and found it to be rather a poor antigen in man, although in a few instances definite amounts of precipitin and protective antibody were produced.

Gonococcus

The antigenic classification of the *gonococcus* has proved exceedingy difficult. Each of eight strains studied by Stokinger *et al.* (103) contained one or more antigens not present in other strains, and no classification into types or groups could be made.

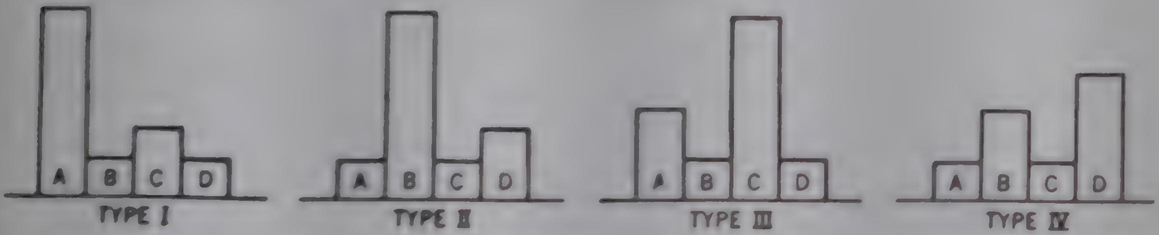

Fig. 15. Hypothetical antigenic structure of *Meningococci*, supposed to depend upon the presence of varying amounts of four antigens, A, B, C, and D (107).

Influenza Bacillus

The *influenza bacillus* (*H. influenzae*), when in the "smooth" form, has been classified into two antigenic types A and B (88) on the basis of agglutination reactions, and precipitin reactions due to a soluble specific substance, presumably a capsular carbohydrate. When the smooth forms are changed to the "rough" variants, the specific soluble substance is no longer produced, and the variants do not conform to this classification.

Whooping Cough Bacillus

All recently isolated strains of the *whooping cough bacillus* (*H. pertussis*) seem to be in the smooth state, and to belong to the same antigenic type.

Brucella

It was postulated by Wilson and Miles (113) that *Brucella* organisms contained two antigens, A and M. The supposed antigenic constitution of *Br. suis* and *Br. melitensis* is shown in Figure 16. The observations of Miles (74) on rates of agglutination of these bacteria are consistent with this picture.

Substances corresponding to the hypothetical A and M antigens have not been isolated. Pennell and Huddleson (87) and Miles and Pirie (75) have isolated complex endoantigens from *Brucella*, amounting to about 25% of the bacterial cell. These contained lipoids and phospholipides, protein-like material, a substance designated as AP, similar to the Boivin antigens, and the polymerized formyl derivative of an amino compound. Both the A and M antigens seemed to be present in the AP substance.

The toxic antigenic complex of brucella organisms seems (26, 75) to consist of a compound of a phospholipde (PL) and an arginine-containing protein (S). The whole complex is represented by the letters PLAPS. There is reason to believe this complex is on the surface of the cell.

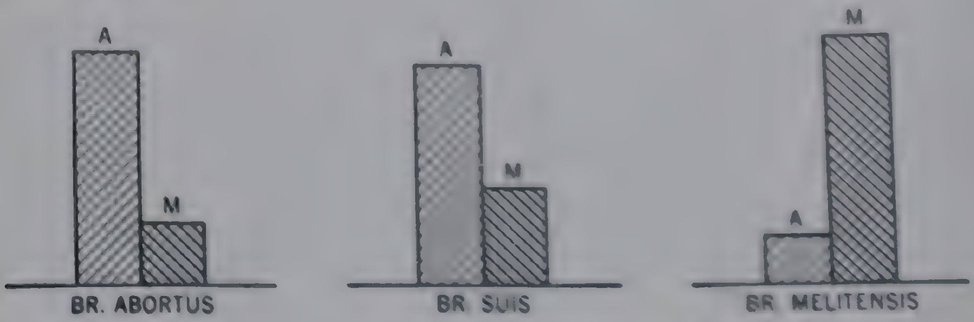

Fig. 16. Hypothetical antigenic composition of three species of *Brucella* (107).

Friedländer Bacillus

The immunological behavior of the *Friedländer bacillus* seems to depend on the presence in the cell of a capsular polysaccharide responsible for type specificity, and a nucleoprotein in the body of the cell responsible for species specificity. Julianelle (48) distinguishes three serological types; A, B, and C, and a heterogeneous group, X, of rough strains. The polysaccharides of the types A, B, and C have been isolated and studied (Table XVIII). As already mentioned, type B polysaccharide is similar to that of type 2 pneumococcus (8).

Dysentery Bacilli

The antigenic behavior of the *dysentery bacilli* is complex, but has also been explained on the basis of the presence of various amounts of a number of different antigens (7). The work of Morgan on the antigenic complex isolated from the Shiga bacillus has already been referred to (Chapter III). Morgan and Partridge (78) considered this to be a homogeneous substance, which they represent as ABC, where A stands for a phospholipide, B a polysaccharide, and C a "polypeptide-like" substance now claimed to be a conjugated protein. Injection of ABC gives rise to antibodies similar to those produced by the intact organisms, together with a little precipitin for C. More recently, Morgan and Partridge (78) have succeeded in removing the phospholipide from this complex, leaving a complex of polysaccharide and polypeptide-like material which is strongly antigenic and gives rise to the Shiga heterogenetic antibodies (see page 141) as well as the Shiga agglutinins and precipitins. By the use of alkali, or preferably phenol–alcohol methods, the carbohydrate has been isolated (79). A alone and AB are not antigenic. Injection of C into rabbits gives rise to antibodies which precipitate C or BC. An antigenic complex BC can be regenerated from isolated B and C. Since attempts to produce antibodies by injecting the polysaccharide adsorbed on nonspecific substances were unsuccessful, it was concluded that the polysaccharide determines the specificity and the "polypeptide-like" component makes the complex antigenic. Recent work suggests that C is really composed of a simple protein and a prosthetic group containing phosphorus, and that the special antigenic property is due to the prosthetic group.

Weil (110) has written an excellent review of the progress in the study of bacillary dysentery. See also Neter (82) and Steabben (102).

Anthrax Bacillus

The specific substance of the body of the *Anthrax bacillus* has been found to be a colloidal polysaccharide constructed of *d*-glucosamine, galactose, and acetic acid (47, 108). The acetic acid is at least partly in the form of -*N*-acetyl. Thus the constituents of this carbohydrate are the same as those of type 14 pneumococcus and correspond in part to those of the human blood group A substance. Ivánovics (47) found that horse antianthrax sera agglutinated type 14 pneumococcus, although rabbit antisera did not. Pneumococcus type 14 would not absorb all precipitins for the anthrax polysaccharide from horse antisera, however. Horse antianthrax sera did not react with group A substance, nor did they agglutinate human group A cells specifically.

The specific substance of the capsule of anthrax, unlike those of many other microorganisms, is not a polysaccharide, but a protein-like substance (47), a polypeptide composed apparently of the single amino acid, $d(-)$-glutamic acid, which is thus in the "unnatural" form (the natural form being $l(+)$-glutamic acid, where l indicates configuration, and the (+) the actual rotation). It is possible that the unnatural form of the amino acid in this substance, making it resistant to the natural enzymes, may be connected with the virulence of the organisms (47).

Salmonella Bacilli

The *Salmonella bacilli* are mostly flagellated, and can be subdivided both on the basis of the antigens of the body of the organism (*somatic*, or O antigens) (94) and those of the flagella (*flagellar*, or H antigens). The use of these letters arose from abbreviations of the German expressions "*Hauch*" and "*Ohne Hauch*," referring to colonies which possessed a "*Hauch*" (breath or emanation) around them, because of the motility of the organisms, and colonies which had sharp boundaries, since the component organisms were nonmotile, and the colonies were thus without "*Hauch*." The H type often presents a thin spreading growth over the surface of the agar plate, as contrasted to the isolated colonies of the O forms (26). The H antigens are of two kinds: those shared by a number of species, or types, and those peculiar to the particular species, or type, or shared by only a few species or types. Many of the species or types are *diphasic*; that is, at one stage of a culture the specific flagellar antigens may be found (specific phase), while at another stage the group antigens may be present (group phase). Any given culture of such organisms may consist entirely of one or the other of the phases, or may contain both. A bacillus in one phase usually keeps the same phase for a number of generations, but is always capable of giving rise to the other phase, and as a matter of fact the antigens of either phase may occur in various types, although the "specific" antigens are generally restricted to a smaller number of types (26).

The somatic (O) antigens are designated by Roman numerals, and according to Dubos (26) like O antigens of the smooth variants of other Gram-negative bacteria, help protect the bacillus against the body's defenses, thus contributing to virulence. This explains why anti-O agglutinins seem more important in resistance than do anti-H agglutinins (111).

A number of methods of releasing the specific carbohydrate in a fully antigenic form have been devised (26). These methods include extraction with trichloracetic acid (12), phenol (76), diethylene glycol (80), etc.

The species flagellar (H) antigens are represented by small letters, and more than 27 have been found; the group flagellar antigens are given arabic numerals. The antigens found in certain important strains are shown in Table XIX, taken from the scheme of Kauffman and White (see 107).

From the table it can be seen that if we were to confine ourselves to the O antigens (which could be done by first extracting the bacteria with hot alcohol to remove or inactivate the flagellar antigens), we should find that an immune serum to *S. schottmuelleri* agglutinates all strains having somatic factors IV or V, and that if such a serum were absorbed with *Salmonella* (Reading type), we should then get agglutination only with organisms possessing V, like *S. typhimurium*.

Freeman (32) obtained the antigenic complex of *S. typhimurium* by alcohol and ammonium sulfate fractionation. The product consisted of a mixture of at least two substances having sedimentation constants of about 57 and 80, respectively. Hydrolysis by acetic acid gave (*a*) a specific polysaccharide (69%), (*b*) an insoluble conjugated protein (16%), (*c*) a mixed lipide fraction (3–4%), and (*d*) a nonantigenic alcohol-soluble carbohydrate (about 8%).

TABLE XIX

SOMATIC AND FLAGELLAR ANTIGENS IN CERTAIN *Salmonella* STRAINS

Group	Species	O antigen	H antigen	
			Specific	Nonspecific group
B	*Salmonella schottmuelleri*	IV, V, (XII)	b	1, 2
B	*Salmonella typhimurium*	IV, V, (XII)	i	1, 2, 3
B	*Salmonella* (Reading type)	IV, (XII)	e, h	1, 4, 5
C	*Salmonella choleraesuis*	VI, VII	c	1, 3, 4, 5
C	*Salmonella* (Newport type)	VI, VIII	e, h	1, 2, 3
D	*Salmonella enteritidis*	IX, (XII)	g, o, m	—

The chemistry of the *Salmonella* bacteria was reviewed in 1943 by Bornstein (13). Serological relationships are discussed by Saphra and Wassermann (91).

5. THE Vi ANTIGEN

Felix and Pitt (29) found that different smooth strains of typhoid bacilli differed in their agglutinability by O antiserum, and that the more virulent were the less agglutinable. This was later shown to be due to the presence in the virulent strains of a special, very labile antigen, which Felix called the Vi antigen (28). Only strains which possess maximal amounts of O and Vi have the highest degree of pathogenicity of which the typhoid bacillus is capable. The Vi antigen is so labile that tests for it were originally only carried out with living suspensions. Peluffo (86), however, has reported results which suggested that the stability of the Vi antigen to other destructive agents is greater than previously supposed, and he believes that when dehydrated it resists heat fairly well. Felix has reported being able to prepare vaccines killed with alcohol which are still antigenic for Vi.

The somatic O antigen has been identified by Boivin and Mesrobeanu (12) with the carbohydrate–lipide complex isolated by them (see Chapter III), and the Vi antigen with another distinct carbohydrate–lipide antigen of the same class of substances.

The fact that anti-Vi antibody agglutinates cells containing the Vi antigen and protects animals against experimental infection with Vi organisms indicates, according to Dubos (26) that this antigen is present at the cell surface. The O antigen is considered to be nearly equally superficial, but the Vi antigen is evidently more so, as its presence can prevent agglutination of O cells by anti-O serum (2). Almon (2) believes that organisms containing the Vi antigen multiply faster in the body than do those lacking it, because of the protective action the Vi antigen exerts against the phagocytes.

A source of error in investigations on bacterial polysaccharides has been pointed out by Sordelli and Mayer (100) and Morgan (76). It consists in the fact that a constituent of the medium on which bacteria are often grown is agar, itself a carbohydrate, and capable of producing antibodies when in association with bacteria, particularly with Gram-negative bacilli, or with conjugated protein extracted from them. The reactions of such antibodies have probably misled certain workers into reporting false cross reactions of some bacterial carbohydrates (60).

6. Virus Antigens

It is difficult to make any definition of a virus which is free from objection. The word "virus" was originally used to mean a poison, such as snake venom. It was later used to mean any infectious disease-producing entity, irrespective of its nature, and only lost this significance as the bacterial causes of certain diseases became clear. Its use persisted in those diseases where the causative agent remained mysterious, and as the virus diseases remained longest in this class, we finally have the meaning restricted to this class of agents. For a discussion of the nature of viruses, see Stanley (101). The simplest appear to consist of protein and nucleic acid, and the most complex, of a number of materials, indistinguishable chemically from those found in bacteria. The evidence available at present indicates that the various purified preparations which have been obtained, even the crystalline preparations of plant viruses, actually represent the active agent itself, substantially free from impurity.

It is now thought that the inclusion bodies found within cells of the host in virus diseases consist essentially of the active agent, in some cases perhaps aggregated into colonies. When separated and washed, these are known as "elementary bodies." Not all viruses, of course, appear thus in visible form inside the cells of the host.

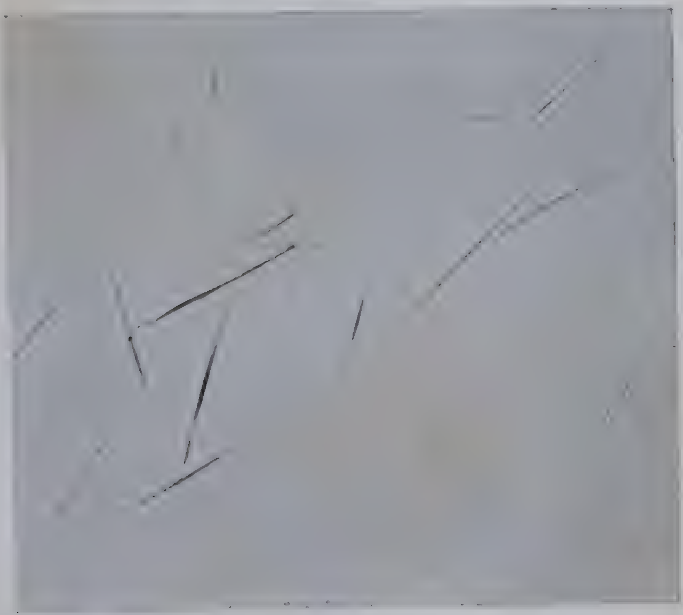

Fig. 17. Crystalline tobacco mosaic virus (× 750) (*courtesy of Dr. W. M. Stanley*).

Originally viruses were characterized by their ability to go through pores which hold back bacteria (and therefore referred to as "filterable viruses"), and by the facts that they could not be seen under the microscope, and that they grew only in the presence of living cells. It is now known that they vary in size very much, and the elementary bodies of the vaccinia virus, for example, can be seen under the microscope.

Tobacco mosaic virus appears to be composed only of protein and nucleic acid. In 1935 Stanley (101) isolated this virus in a form which he described as crystalline (see Fig. 17). The particles of virus which compose such crystals have a diameter of about 150 Å., with a length of about 2800 Å. According to Bernal and Fankuchen (11) these particles have themselves some of the attributes of protein crystals, although these workers think the great length of the particles as isolated may be an artifact. They suggest that the tobacco mosaic particle may be made up of smaller subunits, and that these may be made up of smaller "sub-subunits," definitely of a smaller order of magnitude than crystalline protein molecules, about 11 × 11 × 11 Å. However, it is an accepted fact that, to date, virus activity has not been found to be associated with particles smaller than the 150 × 2800 Å.

Knight and Stanley (53) have estimated some of the amino acids of certain plant viruses, which seem to be among the simplest in chemical make-up. Tobacco mosaic and aucuba mosaic viruses, which are closely related immunologically, were found to be only slightly different, but cucumber virus 4, which does not cross-react serologically with these, contains histidine and much less tryptophan.

Protein, nucleoprotein, phospholipide, neutral fat, and carbohydrate have been identified in preparations of purified elementary bodies of vaccinia (68, 98, 99). It is possible that considerable amounts of normal tissue constituents are still present in the most highly purified preparations of this and certain other animal viruses (50). It is therefore doubtful if the analytical data thus far obtained are characteristic of the virus itself.

Injection of vaccinia virus produces antibodies (including neutralizing substances), against a heat-labile (L) and a heat-stable (S) soluble substance, an agglutinin designated "X", and an antibody against the nucleoprotein. The L and S antibodies are considered by Smadel and Shedlovsky (99) to represent two antigenically distinct parts of a single protein, which has a molecular weight of about 240,000, and an axial ratio of about 30:1.

A good many studies have been made on the virus of influenza, especially during World War II. The early results were not too consistent, for Chambers *et al.* (20) calculated that the particles of influenza A are about 110 Å in diameter, whereas Sharp *et al.* (95) estimate about 1150 Å. This discrepancy has recently been resolved and there is now general agreement that the size of influenza virus is about 1000 Å (101a).

At least two strains (A and B) of influenza virus are known, and Burnet believes (18) that the virus which caused the great 1918 world epidemic was different from either of these.

7. Antigens of Parasites

Parasites also contain antigenic proteins (72) and polysaccharides. For instance, Melcher and Campbell (73) isolated in "a reasonably pure chemical condition," a polysaccharide from *Trichinella spiralis*, which gave immunologically specific reactions when tested on *Trichinella*-infected rabbits.

8. Antigens of Animal Cells

In animal cells also a variety of antigens, including proteins and carbohydrates, are found. These probably play, in parasitic infection, for instance, roles analogous to those of the corresponding antigens of bacteria. This question will be taken up briefly in Chapter IX. An important class of animal cell antigens determine species and individual differences in the erythrocytes. These are discussed in the next chapter.

9. Structure of Cell Antigens

The antigenic relationships described above suggest that bacterial and animal cells consist of a "mosaic" of antigens. In view of the known complexity of these cells, this view has an inherent plausibility, and it will be noted that in many cases it has been supported by the isolation of one or more of the hypothetical antigenic "factors." However, in other cases (for example, Brucella, Shiga) it has not proved possible to separate out chemically distinct substances corresponding to the hypothetical antigens, and from other work (see page 37) we know that one chemical complex can give rise to more than one distinct antibody. A number of the recent workers (Boivin, Raistrick, Morgan) have emphasized that there is a certain risk in separating a cell arbitrarily into proteins, carbohydrates, lipides, etc., when it is entirely possible that the living cell substance is essentially a complex, labile combination of all of these elements simultaneously. The particular compound which is obtained by breaking down the cell might depend on which constituents happen to emerge in combination with each other in the final product. This would account for the variety of compounds which can be isolated by different procedures.

Thus Morgan (77) says that it is probable that bacterial antigen as it exists in a bacterial cell is not a simple compound of rigid chemical constitution, but consists of a labile molecular aggregate, possessing some essential component—such as polysaccharide—of definite chemical structure and fixed composition, which determines the strict specificity of the antigen, together with other loosely bound constituents which endow the essential component with antigenic properties.

When an antiserum to the cells of one species is tested against those of a related organism, and a cross reaction is obtained, it is natural to assume that this is because the cells of the second species possess a chemical substance in common with the homologous cells. If the serum is absorbed with the heterologous cells, and, as is usually the case, antibody is left which still reacts with the homologous cells, it is again natural to assume that the antiserum contained antibodies to two (or more) chemical components in the homologous cells, only one (or a few) of which was common to the two sorts of cells. This has probably been the prevailing view among immunologists, and it is illustrated by the following scheme, taken from Landsteiner (59), which is meant to indicate the antigenic composition of several individuals. Columns 1 and 2 represent the antigenic components of two individuals of the same species, column 3 those of a related, column 4 of an unrelated, species.

A	A	B	D′
B	B	C′	L
C	C	F	M
D	D	G	N
E	F	H	O
.	.	.	.
.	.	.	.

There is no doubt that this simple concept is often correct, and serves to explain most or all of the individual blood differences in man and probably the lower animals (Chapter V). In these cases the characters causing the differences have often been shown to be unit characters, inherited according to the Mendelian rules.

The reader will have noticed in the above pages, however, how often this sort of explanation has been invoked by the various workers to explain the antigenic variation in different bacteria. As Landsteiner (59) has pointed out, it is not likely that the assumption of different distinct chemical structures to explain the observed reactions is invariably correct. We have seen that an antibody may react with several different substances of related constitution, and different immune sera may react with the same substance. Therefore, as Landsteiner says (59): ". . . it is not requisite that an antigen which reacts with several antibodies has an equivalent number of binding substances or distinctive, specific groups. Neither is the fractionation of antisera by partial exhaustion with heterologous antigens by itself unfailing proof for this conclusion, and an alternative explanation is to be considered, namely the formation of divers antibodies in response to a single determinant group."

So in some cases we should rather suppose, not that the related cells contain one or more chemical compounds in common with the homologous cells, but that they contain similar, but not identical, chemical structures. This might well be the explanation, for instance, of the observations on *Brucella* and the Shiga bacillus, mentioned above. It is likely that it explains some of the peculiar observations made in the study of the Forssman hapten (see above). Landsteiner says (59): "The incomplete absorption of sheep hemolysins by human blood A (Schiff and Adelsberger) and of lysins in group-specific anti-A sera by sheep blood can be explained if one assumes that antibodies of different reaction range are formed through the action of single determinant structures similar but not identical, in each of the two antigens, less specific antibodies being responsible for the cross reactions." See page 37.

Toxins have been discussed in Chapter III.

SUMMARY

(*1*) Closely related species may often be differentiated, not only by the precipitin test, but also by agglutination tests. Two examples are hemagglutination of animal red cells and bacterial agglutination using absorbed sera. (*2*) The Forssman hapten is probably not a definite chemical entity, but a collective serological term. The original observations were that injection of minced guinea pig organs into rabbits produced strong lysins for sheep cells, indicating some common antigenic component. The Forssman hapten, in the wider sense is found widely distributed in the animal, and even the plant, kingdom. Its distribution tends to correlate with taxonomic relationships. Purified Forssman substance from horse kidney has been found to be a nitrogen-containing carbohydrate. Other serological cross reactions between tissues of species not very closely related have been found. The Forssman hapten seems to require the presence of a protein or mixture of proteins ("Schlepper") such as pig serum, or combination with suitable adsorbants to make it antigenic. The Forssman activity seems to be associated in tissues with material of high molecular weight. (*3*) Cell antigens which give rise to agglutinating antibodies for the cell would seem to be localized at, or near, the surface. Different antigens in morphologically different parts of the cell have been demonstrated in bacteria, and also in certain animal cells such as spermatozoa. (*4*) Bacterial species may often be subdivided into types by means of serological reactions. Bacteria in general have long been classified into Gram-positive and Gram-negative types. Dubos believes the positives have a protein–ribonucleate complex at the cell surface. (*a*) Pneumococci may be classified into a large number of types on the basis of antibodies to their capsular polysaccharides. The capsular polysaccharides of pneumococci of types 1, 2, 3, and 8 are made up from molecules of glucose and glucuronic acid. The structural unit of the type 3 polysacchairde is now completely known. These polysaccharides have molecular weights of the order of 100,000 or higher. The substance causing the transformation of rough (nonencapsulated) into smooth (encapsulated) pneumococci has been found to be a highly polymerized viscous form of desoxyribonucleic acid. (*b*) The hemolytic streptococci have been classified into types, by means of the precipitin reaction, by Lancefield, who distinguishes a species-specific nucleoprotein, a group-specific carbohydrate (C), and two type-specific substances (M and T). Many types have been found. (*c*) Staphylococci contain polysaccharides which enable at least three types to be distinguished. (*d*) It is customary to classify diphtheria strains into mitis, intermedius, and gravis. (*e*) Serological methods have not been so successful in differentiating strains of acid-fast bacteria. The active principle of tuberculin has been found to be a protein. (*f*) The meningococcus has been classified into various types on the basis of agglutination reactions. (*g*) The antigenic classification of the various strains of gonococcus has proved difficult. (*h*) The influenza bacillus has been classified into types A and B, which seem to have different capsular polysaccharides. (*i*) Types have not been established in *Hemophilus pertussis*. (*j*) The toxic antigenic complex of brucella organisms seems to consist of a phospholipide (PL), and an arginine-containing protein (S). A lipide–protein like material (AP) has also been found. The whole complex is thus PLAPS. (*k*) The Friedländer bacillus has been separated into three types A, B, and C, and a heterogeneous group. (*l*) From dysentery bacilli a substance ABC (A = phospholipide, B = polysaccharide, C = phosphoprotein) has been extracted. (*m*) The anthrax bacillus has a colloidal polysaccharide in the body, and a capsule which is a protein-like polypeptide. (*n*) Salmonella bacilli are mostly flagellated, and contain somatic (O) antigens in the body of the cell and flagellar (H) antigens in the flagella. There are two kinds of H antigens, one kind common to larger numbers of organisms. The latter are given arabic numerals, the former small Roman letters. Different cultures may vary in which antigens they display and are thus called diphasic. (*5*) The Vi (virulence) antigen of certain salmonella and typhoid has been thought to be a carbohydrate–lipide complex like the O antigen isolated by Boivin, but different from it. It is rather labile. Antibodies to agar have sometimes caused complications in investigations on bacterial polysaccharides. (*6*) Viruses were originally characterized by their ability to go through pores which held back bacteria, and were thus "filterable." They vary in size and complexity, and tobacco mosaic virus appears to consist only of protein and nucleic acid. It is possible that some normal tissue components are still present in all the animal viruses thus far studied. At least two strains, A and B, of the influenza virus have been found. (*7*) Parasites (e.g., *Trichinella*) also contain antigenic proteins and polysaccharides. (*8*) Animal cells contain distinct antigens, such as the blood group substances discussed in Chapter V. (*9*) The cell is a complex mosaic of proteins, lipides, polysaccharides, etc., and therefore different treatments may separate different complexes from it. Cross reactions between organisms do not necessarily show the presence in both of the *same* substance, although this may be correct in some cases. Sometimes it proves impossible to isolate any pure compound corresponding to a hypothetical antigen.

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CHAPTER V

BLOOD GROUPS

From what was known of immunology in the last century, it would probably have been supposed that reactions which could distinguish individuals of the same species would be much weaker than those differentiating individuals of different species, and it might have been thought more likely that such reactions would not occur at all. The actual facts are otherwise; it is often possible to distinguish serologically individuals of the same species, and the differences are quite likely to be strong and well marked. In some cases, as in chickens and cattle, large numbers of individual blood differences exist, so that almost every individual tested is found to be different from every other, but in most species thus far examined the number of readily detectable differences is limited, and individuals fall into types, or groups.

1. GROUP SUBSTANCES

The four groups into which human blood can be divided are well known on account of their importance for blood transfusion. These groups, discovered by Landsteiner (40), depend on the presence or absence in the red blood cells of the individual of two chemical structures, called A and B. The chemical nature of these substances has not been extensively investigated, and their exact chemical nature is still not well known. If we can draw any conclusions from studies on the serologically related A substance in hog stomach (43, 66, 93, 94), and the B(+A) substance in horse stomach (95), carbohydrates form a significant part of the complex; small amounts of amino acids (15) have been found. Alcohol extracts of human erythrocytes, when mixed with proteins, will immunize specifically, and show weak activity *in vitro*. More highly purified products are water soluble and nonantigenic or weakly antigenic. Freudenberg *et al.* (25) have also worked on the A substance.

The chemical composition of preparations of A substance from human sources and from animal sources is given in Table XVIII.

The A substance has been found to be related to the Forssman hapten. Some rabbit sera against sheep cells will agglutinate human cells containing A, and antisera against human A cells will even more frequently lyse sheep cells (see Chapter IV, closing paragraphs.)

The specific substance of group B has been isolated (93) from the gastric juice of human individuals of group B.

We might be tempted to consider the O characteristic of human blood as merely the absence of A and B, but sera reacting specifically with O bloods (and to some degree with A_2) can be obtained, as by absorbing cow serum with A_1 or A_1B blood. Precipitins acting on the O substance have been obtained (14). We therefore seem to be justified in assuming the existence of a specific "O substance." Human erythrocytes of all types evidently contain carbohydrate groupings related to the type 14 pneumococcus polysaccharide, since horse antipneumococcus type 14 antisera agglutinate human bloods (2, 22) (see Chapter IV). Witebsky and Klendshoj (94) reported the isolation of a carbohydrate with O properties from the gastric juice of persons of group O.

TABLE XX

THE LANDSTEINER BLOOD GROUPS

International nomenclature	Structure in cells	Agglutinin in serum
O	—	Anti-A + anti-B
A	A	Anti-B (β)
B	B	Anti-A (α)
AB	A + B	—

2. BASIS OF THE FOUR CLASSICAL GROUPS

The group-specific substances A and B may be present in the erythrocytes of an individual either singly or both together, or both may be absent. This makes four combinations, determining the four blood groups, as is illustrated in Table XX.

3. AGGLUTININS

It has been shown (37, 41) that the substances A and B, as they occur in the cell, are antigens, and immune agglutinins can be prepared for them by injecting rabbits with human erythrocytes of groups A and B and absorbing out undesired agglutinins directed towards other features of the cell. Since the substances A and B can give rise to agglutinins, we may refer to them as agglutinogens. These immune agglutinins have certain advantages for theoretical, and possibly for practical, investigations (5). Morgan (65) also showed that artificial complexes of (hog) A substance with the conjugated proteins from the Shiga bacillus (page 128) would produce, in rabbits, powerful and specific anti-A sera. By use of such agglutinins, the presence or absence of A and B can be ascertained, and the blood group determined.

However, it is not necessary to rely solely on these artificially produced agglutinins, for as will be seen from Table XXI, agglutinins are present in normal human serum. The distribution of these agglutinins exhibits a regularity, known as Landsteiner's rule, which has been stated by Landsteiner (42) as follows:

TABLE XXI

DETERMINATION OF BLOOD GROUP OF UNKNOWN CELLS

Serum	Agglutination			
Cells + anti-A (= normal human B serum)	−	+	−	+
Cells + anti-B (= normal human A serum)	−	−	+	+
Group	O	A	B	AB

“. . . the serum regularly contains the agglutinins active for the absent agglutinogens . . . corresponding agglutinins and agglutinogens do not coexist in one blood. . . .”

Antibodies in the serum of an individual for antigens of other individuals of the same species are called isoantibodies; these agglutinins evidently belong in this class.

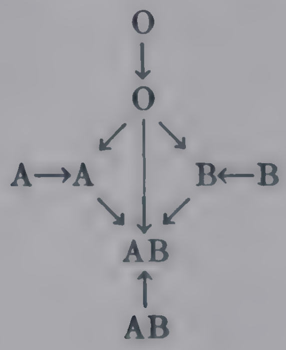
The blood groups were discovered by means of these normal *isoagglutinins*, and it is still customary to use them in routine determination of blood groups, although in some cases the human sera available are not as powerful as could be desired. Probably the best method of routinely obtaining strong and specific grouping reagents is the method of Witebsky, Klendshoj and McNeil (95) of injecting purified A and B (+A) substances (from hog and horse, respectively) into human volunteers of groups A and B. This procedure seems entirely safe, and gives good sera in about half of the volunteers. For grouping unknown bloods it is sufficient to use human sera of groups A and B only, as will be seen from Table XXI. Groups can also be determined, if both serum and cells are available, by using serum and cells of group A or group B. The reader can easily work out the method of doing this for himself (see 76).

The exact mode of origin of the normal isoagglutinins is still not settled, but it has been shown that the human A and B antigens will produce immune agglutinins in human beings, if blood containing one agglutinogen is transfused into individuals without it (87). Animal A and B substances are also antigenic in man (see above). Normal hemolytic antibodies (isohemolysins) are found in many bloods, especially if the tests are made with erythrocytes which have been stored for a time. Their possible occurrence depends on the blood group in the same way as does that of the isoagglutinins.

The detailed technic of blood group determination will be discussed in Chapter XI.

4. Importance in Transfusion

From a scheme such as that of Table XX, it is easy to see why it is essential to determine the blood groups before performing a blood transfusion. Since all human sera, except group AB, contain agglutinins for A or B or both, introduction of a blood containing agglutinogens capable of reacting with an agglutinin in the donor's plasma is likely to lead to agglutination, with resulting disaster. It is generally considered that the effect of the agglutinins of the recipient on the cells of the donor is most to be considered, as the agglutinins of the donor are diluted, and may also combine with soluble group substances in the serum of the recipient, and thus usually fail to exert harmful effect. For this reason the practice grew up of using for a donor any person whose cells could not be agglutinated by the serum of the recipient. This gave the transfusion possibilities shown below, where the arrows indicate the direction of transfusion.



It will be noted that group O could give, in theory, to each of the other three groups, and thus the popular but somewhat misleading term "universal donor" arose. In actual practice it has been found best to use for a donor a person of the same blood group as the recipient, and the so-called "universal donor" is now used only in great emergencies, or after neutralization of the agglutinins present by the addition of A and B substances (95). Large amounts of group O blood were however flown to the fighting fronts during World War II.

It has been found necessary also to test the cells of the donor against the serum of the recipient, and if possible, vice versa, in order to guard against incompatible reactions which sometimes occur, even though the groups seem to be identical. The explanation of these unexpected incompatibilities probably rests on occasional incomplete or weak development of an agglutinin, leading to errors in grouping, and sometimes on the presence of agglutinogens and agglutinins not part of the classical scheme (see below).

A number of unpleasant and dangerous reactions following transfusion have recently been shown to be due to antibodies to a blood factor designated as Rh (48) (see page 190).

5. Inheritance

The four classical blood groups are inherited according to Mendelian rules. Their heredity has been shown (3) to depend on three genes, all capable of occupying the same locus on the chromosome which carries them (allelomorphic genes). These were designated by Bernstein as A, B, and R. The symbol R was later changed to O.

Geneticists have shown that the hereditary characters of animals and plants are determined by units localized in submicroscopic structures called "genes." These are localized in small but microscopically visible rod-like bodies that occur in the nuclei of the cells of which plant and animal bodies are built up and by which they are propagated. These rods are called "chromosomes," and are observed to occur in pairs. A chromosome may carry a number of genes but only one of a kind. One member of each pair of chromosomes carries at each point of its structure a gene corresponding to the one at that same point in the other member of the pair.

During the formation of the cells concerned in reproduction (sperm and ova), these pairs of chromosomes separate, and each sperm or ovum contains only one of each kind of chromosome, and forms an exception to the general rule that each cell contains a pair of each kind. Figure 18 may assist in making this clear.

In the very numerous experiments on many varieties of living things propagated by sexual reproduction, it has been observed that hereditary characters occur in the individual in pairs, whose relationship is shown by breeding experiments, just as this chromosomal theory would demand. The classical human blood groups are inherited by such a mechanism. They are predetermined by the presence or absence in the chromosomes of two factors (or genes) called "A" and "B." Absence of A and B is indicated by "O." Since each body cell has a pair of chromosomes, each of which carries or fails to carry one of these factors, an individual's genetic constitution may be represented by AB, AA, AO, BB, BO, or OO, where O represents the absence in the chromosome of either the A or B factor at that point. From this it is easy to see that the individual whose genetic formula (genotype) is AB will exhibit both of these factors in his blood (and other) cells, while the AA individual will have only the A characteristic, and the OO will have neither A nor B. The thing that is tested for in the blood cells is not the gene itself, but the substances A and B produced under the specific influence of the inherited genes. The fact that the AO individual has only one dose, so to speak, of the A factor seems to make no practical difference, and his blood is routinely indistinguishable, by direct test, from that of the AA individual.

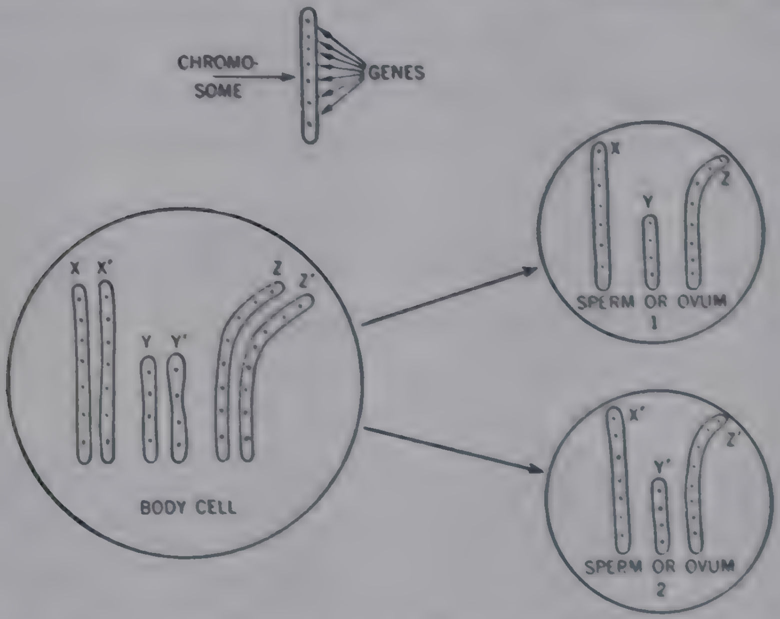


Fig. 18. Separation of chromosome pairs in the formation of gametes.

This means that we have but four types of blood (phenotypes), O, A, B, and AB, routinely distinguishable from each other by direct laboratory tests. These types or groups are given in Table XXII, together with the corresponding designations in the two other (obsolete) systems of nomenclature which were applied before the genetics of blood grouping was understood. These earlier systems of naming, before the late war, were still unfortunately used to some extent in certain hospitals.

Knowing the way in which mature reproductive cells ("gametes") of each sex emerge with only one chromosome of a pair, and consequently only one gene of a pair, we can predict mathematically the way in which the blood groups will be inherited. When sperm and ovum merge it is readily understood that the resulting cell contains its set of pairs of chromosomes; each parent has contributed one member of each pair.

TABLE XXII

DESIGNATION OF THE BLOOD GROUPS, AND THEIR GENETIC FORMULAS

International name (phenotype)	Jansky name[a]	Moss name[a]	Genetic constitution (genotype)
O	I	IV	OO
A	II	II	AA or AO
B	III	III	BB or BO
AB	IV	I	AB

[a] Now obsolete.

A man of group AB produces two types of sperm, one containing the factor A and one containing the factor B. These are produced on the average in equal numbers. A woman of group AB will produce two types of ovum, one containing A and one containing B. If such a man and woman mate, it will be a matter of even chances whether an A sperm fertilizes a B or an A ovum, and whether a B sperm fertilizes a B or an A ovum, so that three types of offspring could be produced, AB, AA, BB, BA. (The AB and BA offspring will be just alike, since each will have both substances in his blood.) So the percentage of offspring, if we observe a statistically large enough number of such matings (or of children of one such mating), will be 25% A, 25% B, and 50% AB. It is conventional in genetic literature to illustrate such a process of reasoning about the results of a mating by the checkerboard diagrams shown in Table XXIII. (Either factor shown on the left may combine with either of the two at the top of each diagram.)

When we come to a mating involving an individual ("phenotype") of group A or B we must remember that there are really two genotypes in each group; e.g., one whose genetic constitution is AA (homozygous), and another whose constitution is AO (heterozygous). The first will produce only one kind of gamete (or sexual cell), all containing A, while the other will produce two types of gamete, 50% containing A and 50% not containing A.

TABLE XXIII

DIAGRAMMATIC EXAMPLES OF THE HEREDITARY TRANSMISSION OF THE FACTORS THAT DETERMINE CERTAIN BLOOD GROUPS

(I)
Mating AA × AA

	A	A
A	AA	AA
A	AA	AA

(II)
AA × AO

	A	O
A	AA	AO
A	AA	AO

(III)
AO × BO

	B	O
A	AB	AO
O	BO	OO

(IV)
OO × AB

	A	B
O	AO	BO
O	AO	BO

PROGENY

(I)	(II)	(III)	(IV)
100% A	100% A	25% O 25% A 25% B 25% AB	50% A 50% B

On the top of each diagram are designated the factors possessed (and transmissable) by one parent; on the left, those of the other; the letters within the horizontal lines show the possible results in progeny. Diagrams of all the 21 possible types of mating are tabulated in (92).

Four of the 21 possible kinds of mating are shown in Table XXIII. The third shows how a child's group may differ from that of either parent; the fourth shows that an O parent can not have an AB child, that an O child can not descend from an AB parent, and that an AB child can not descend from an O parent. The reader will be able to work out readily the other 17 possible types of mating for himself, and thus verify Table XXIV.

6. APPLICATIONS TO LEGAL MEDICINE

Disputed Paternity

From the above it will be clear how we may apply our knowledge of the inheritance of the blood groups in testing cases of disputed paternity. If a man is accused of being the father of an illegitimate child, we may determine whether the group of the child is such that, according to Table XXIV, the man could not be the father. In Table XXV are collected all the combinations which make this possible.

Naturally, if the groups are such that the man *could* have been the father, in the majority of cases we can not state that he was, for there is always the possibility that some other man of the same group (or some other compatible group) is the father. Only in unusual circumstances of isolation would it be possible thus affirmatively to fix paternity on a man. In most of the states, at least, where blood-grouping evidence is admitted by law in such cases (New York, New Jersey, Ohio, Wisconsin, Maine, Maryland, and South Dakota) it is provided that the evidence shall not be introduced unless the paternity of the man is *excluded*.

TABLE XXIV

BLOOD GROUPS OF OFFSPRING POSSIBLE OR IMPOSSIBLE FROM ANY MATING COMBINATION

Alleged father	Known mother	Possible children from their mating	Children not possible from their mating. Decisive for nonpaternity	Impossible from this mother in any mating
O	O	O	A, B, (AB)	AB
O	A	O, A	B, AB	
O	B	O, B	A, AB	
O	AB	A, B	(O) AB	O
A	O	O, A	B, (AB)	AB
A	A	O, A	B, AB	
A	B	O, A, B, AB		
A	AB	A, B, AB	(O)	O
B	O	O, B	A, (AB)	AB
B	A	O, A, B, AB		
B	B	O, B	A, AB	
B	AB	B, A, AB	(O)	O
AB	O	A, B	O, (AB)	AB
AB	A	A, B, AB	O	
AB	B	A, B, AB	O	
AB	AB	A, B, AB	(O)	O

The letters designate the blood types of the respective individuals (see Table XX). Those in parentheses, in column 4, could not be children of the corresponding mothers (column 2) in any mating. (See Wigmore's complete tabulation of genotypic matings.) Therefore no such child could exist to raise a problem of proof, and these instances are omitted from Table XXV which summarizes the net indications of nonpaternity deducible from these blood groupings.

Disputed Maternity

It is rare that any uncertainty arises over the maternity of a woman and her presumed child. From Table XXIV, it will be seen that in certain cases nonmaternity could be established. Cases have actually been observed in which the woman tried to introduce into a legitimacy case a child not her own.

Interchange of Infants

In case of suspected interchange of infants, if all four parents and the children are available for testing, it can often be shown, if any interchange really occurred, that the blood groups indicate that the correct allocation of the infants is different. This can easily be seen from the tables, and the method has had practical application.

TABLE XXV

COMBINATIONS ALLOWING A MAN TO ESTABLISH NONPATERNITY, OMITTING INSTANCES OF IMPOSSIBLE MOTHER-CHILD COMBINATION

Putative father	Known mother	Known child
O	O	A, B
O	A	B, AB
O	B	A, AB
O	AB	AB
A	O	B
A	A	B, AB
B	O	A
B	B	A, AB
AB	O	O
AB	A	O
AB	B	O

Chances of Establishing Nonpaternity

It is clearly desirable to know approximately how often a falsely accused man may expect to establish his innocence by such tests, and a number of people (see 4) more or less simultaneously calculated the probability of establishing nonpaternity by blood group determinations. Probabilities relating to the white population of the United States are given in Table XXVI.

Determination of Blood Group of Blood Stains

In a number of forensic cases the question of the identity of a blood stain comes up. Let us suppose, for instance, that a suspect is arrested for murder. The crime was one which involved the spilling of blood, and in possession of the suspect a blood stained handkerchief is found. The police believe the stain results from wiping hands or a weapon on the cloth at the scene of the crime, but the accused says it is his own blood, from a nosebleed. It is clearly desirable to check his story.

If we determine the blood group of the suspect, the group of the blood on the handkerchief, and (if possible) the group of the victim, we may possibly find that the group of the blood on the handkerchief is different from that of the suspect, in which case his story is false, and the case against him becomes stronger than ever. It has been found possible to determine the group of dried blood, even when it is quite old, by means of its absorptive power for the agglutinins anti-A and anti-B. A recent summary, and description of technic is given in Schiff and Boyd (76) and in (12).

TABLE XXVI

PROBABILITIES OF PROVING NONPATERNITY WHEN BLOOD GROUP OF WRONGFULLY ACCUSED MAN IS KNOWN (38, 39)

Group	Approximate per cent in the United States	Probabilities
O	45	1/5*
A	42	1/17
B	10	1/7
AB	3	1/2
(If unknown)		(1/7)

* 1/5 = 1 in 5 = 20%.

Material Other Than Blood

It is possible, in fact often even easier than with blood, to determine the blood groups from dried material other than blood, because of the presence of the antigens A and B in other tissues and fluids of the body (see below). This method has been applied to saliva, sperm, etc. The forensic application of both the precipitin test and of blood-grouping tests, and the qualifications of an expert in these technics, have been discussed elsewhere at some length by the present author (8, 10).

7. OCCURRENCE OF A AND B IN THE HUMAN BODY OUTSIDE OF THE ERYTHROCYTES

The antigens A and B are not restricted to the red cells of the blood. They seem to occur in all cells and tissue fluids, except the cerebrospinal fluid, testicle, lens, chorion frondosum of the placenta, hair, compact bone, cartilage, epithelial cells of the skin, and the nails. Table XXVII, taken from Boyd (4), shows the relative amounts in the parts of the body which have been studied quantitatively.

TABLE XXVII

AMOUNTS OF GROUP SUBSTANCES IN ORGANS AND FLUIDS (35, 75)

I. Relative Amounts of Water-Soluble Group Substances in Organs and Feces of Secretors (Inhibiting Doses per Gram)

Organ	Moist weight	Dry weight	Organ	Moist weight	Dry weight
Brain	2-4	26	Lungs	9-22	111
Spleen	4	—	Kidney	29	86
Feces	5	100	Heart	34	107
Aorta	10	24	Oesophagus	200	—
Muscle	10-20	36	Pancreas	180-310	1390
Liver	16	48	Duodenum	400	—
Fat	16	—	Stomach	2000	—
Kidney	16	—			

II. Relative Amounts of Group Substances in Excretions and Secretions, Compared with Erythrocytes (Dilutions which Inhibit Isohemagglutination) (72)

Material	Dilution	Material	Dilution
Saliva	128–1024	Tears	2–8
Sperm	128–1024	Urine	2–4
Amniotic fluid	64–256	Cerebrospinal fluid	0
Erythrocytes	8–32		

8. BLOOD GROUP ENZYME

The group substances (from saliva or food) are largely destroyed in descending the digestive tract. This is not due to the ordinary digestive enzymes, such as pepsin or trypsin. Sterile filtrates of stool suspensions (human and animal) have this action; they lose it after being heated. The O characteristic seems to be destroyed in the same way. Saliva itself has some destructive power, but the activity is bound to the cellular debris if autolysis is prevented by cooling immediately after collecting the saliva.

9. Secreting and Nonsecreting Types

Antigens A and B are not invariably present in the saliva when present in the blood. Their presence or absence in the saliva seems to be determined by a pair of Mendelian genes, designated by S (secretor) and s (nonsecretor) (77). The secreting type is more common; the relative frequencies of secretors and nonsecretors seem to vary in different races. There is evidence that the group substance found in the saliva is actually manufactured in the salivary glands (28).

In the organs of nonsecretors little or no water-soluble group substance is found, but active substances can be extracted with alcohol, and the group specificity of the organs can also be demonstrated by absorption experiments.

Some animals excrete in their saliva (and perhaps in other fluids) substances related serologically to the A and B structures of man (see below).

10. The M and N Types of Landsteiner and Levine

In 1927 Landsteiner and Levine (45) discovered three additional antigens in human erythrocytes. These factors had escaped discovery because of the absence in normal human serum of agglutinins for them, and their consequent failure to bear importantly on transfusion problems. They have proved to have considerable forensic and anthropological importance, however, and M and N have since been extensively studied.

11. Differences from the A, B Blood Groups

The M and N factors differ in a number of important respects from the A and B antigens. In the first place, as already mentioned, isoagglutinins for them are never found in normal human serum (except in very rare cases an anti-M). This means that M and N must be detected by the use of immune agglutinins from animals, as can also be done for A and B (see above).

So far practically nothing is known about the chemical nature of M and N, but they differ in their solubility and extractibility from A and B. Unlike A and B, M and N have not been certainly demonstrated in any of the tissues or body fluids, but seem so far as we know, to be confined to the erythrocytes. Substances closely related to M and N serologically do not seem to occur in lower animals and bacteria, as is the case with A and B.

12. INHERITANCE OF M AND N

The M, N types are inherited as a simple Mendelian pair, without dominance. No person is found without M or N, or both, in his blood, so no type corresponding to group O occurs.

Thus we have the genetic formulas MM, NN, and MN, giving the blood types M, N, and MN. The inheritance will be readily understood on the basis of the same considerations applying to the A, B groups (above), and the results in Table XXVIII can be verified by the reader.

TABLE XXVIII

INHERITANCE OF THE M, N TYPES

Mating	Per cent of children of types		
	M	MN	N
M × M	100	0	0
N × N	0	0	100
M × N	0	100	0
M × MN	50	50	0
N × MN	0	50	50
MN × MN	25	50	25

13. APPLICATIONS TO FORENSIC MEDICINE

Since the laws of inheritance of M and N are exactly known, it is possible to make use of these factors in forensic cases involving relationship, including disputed paternity, disputed maternity, and interchange of infants. From Table XXIX the combinations which will enable a falsely accused man to establish his innocence can easily be seen.

The total *probability* of establishing nonpaternity by the use of the M, N types is about the same as with the A, B groups; the employment of tests for M and N approximately doubles the chance which would derive from the A, B groups alone. See Table XXX.

The results of nonpaternity tests in actual practice have agreed well with expectation. However, since we can not expect that all the accused men are innocent, it is clear that the tests will *not* exonerate the proportion of defendants predicted above. If just half the defendants were guilty, for instance, we should find that instead of establishing the innocence of about one-third of them, the tests would exonerate only about one-sixth. This is roughly what happens in practice, suggesting that about half the men tested were guilty, and half innocent.

TABLE XXIX

COMBINATIONS ALLOWING A FALSELY ACCUSED MAN TO ESTABLISH NONPATERNITY BY M, N TYPES

Accused	Mother	Child
M	M	MN
M	MN	N
M	N	N
N	M	M
N	MN	M
N	N	MN

TABLE XXX

CHANCES OF PROVING NONPATERNITY BY USE OF M, N TYPES (79)

Accused	Chances of proving nonpaternity	
	Per cent	About
M	34.11	1/3
N	41.09	2/5
MN	0	0
In general	18.64	1/5
Combined test, A, B and M, N	33.07	1/3

Determination of the M, N type of dried blood stains has proved much less certain than determination of the A, B groups, although the methods for M are probably reliable enough to be recommended for forensic work. Since M and N do not seem to occur outside the erythrocytes, it is not possible to determine the M, N types from other dried material.

14. ONTOGENETIC DEVELOPMENT OF GROUP-SPECIFIC FACTORS

Agglutinogens A, B, M, and N are detectable in new-born infants, and, after the first few months, in the fetus. The A and B factors, at any rate, seem to increase in strength after birth, and may again become weaker in old age, but they seem always to be strong enough in new-born infants to allow the blood groups to be determined without error if strong sera are used, immune sera being particularly valuable here.

Only about one-half of new-born infants have demonstrable isoagglutinins. The agglutinins in new-born infants are apparently those of the mother; at least the infant never has an agglutinin capable of acting on the mother's cells. These agglutinins disappear shortly after birth and are later replaced by the infant's own agglutinins, so that the group becomes complete in this sense also between the third and twelfth month after birth.

TABLE XXXI

APPROXIMATE FREQUENCIES OF THE A, B BLOOD GROUPS AND M, N TYPES IN THE WHITE POPULATION OF THE UNITED STATES

Group	Per cent	Type	Per cent
O	45.0	M	30.1
A	41.0	MN	47.9
B	10.0	N	22.0
AB	4.0		

15. BLOOD GROUPS AND RACE

Thus far no serological property of the blood has been found which is found present in one race and absent from all others. In view of the enormous amount of mixture which went into making the present human "races," it is not likely that any such character will ever be found. It has been found, however, that the proportion of a population belonging to one or another blood group may vary in different ethnic groups (36). A very large number of studies have been made on the blood group frequencies in different populations. A summary, complete up to June, 1938, has been published (4). Much valuable new data, especially in regard to the new Rh blood factors (see below), has since been published in the *Australian Journal of Medicine*, *Science*, and other journals there referred to.

It has been found that there are certain systematic geographical variations in the frequency of the blood groups and types. The approximate frequencies in the white population of the United States are shown in Table XXXI. It is possible that in the future the blood groups will be recognized as the most valuable single anthropological criterion which we possess at the present time, once anthropologists come to appreciate the unique advantages they offer (6).

If we examine compilations of blood group percentages from other parts of the world we find, as would be expected, that peoples of northern Europe have percentages similar to those of the United States (or properly put, vice versa). In other parts of the world, as in Asia and Australia (aboriginal population) the frequencies may differ considerably from this. In central Asia group B is more frequent, and in Australia B falls to zero. The M, N frequencies show similar, but on the whole less marked variation.

The most graphic way of showing the general world trends in blood groups and types is to plot on a map the frequencies of the genes. If we represent the frequencies of the genes A, B, and O by the letters p, q, and r, it is sufficient to show the values of p and q, since $r = 1 - (p + q)$. For the M, N types the frequency of one gene, say that for N (represented by n) is sufficient, for $m + n = 1$. These gene frequencies can be calculated by the relations:

$$p = \sqrt{\bar{O} + \bar{A}} - \sqrt{\bar{O}}$$

$$q = \sqrt{\bar{O} + \bar{B}} - \sqrt{\bar{O}}$$

$$n = \overline{MN}/2 + \bar{N}$$

where the symbols $\bar{O}$, $\bar{A}$, $\bar{B}$, $\overline{MN}$, $\bar{N}$ represent the frequencies (per cent divided by 100) of these groups in the population. The geographic variation will be noted in the maps showing the gene frequencies p, q, and n (Figs. 19a, 19b, 20).

There has been much speculation as to the cause of these variations in blood group gene frequencies. It would be impossible to discuss this question fully here; interested readers will find a full discussion given by the author elsewhere (6, 10a). The frequency of other human genes shows similar variation.

16. Determination of Blood Groups in Human Remains

The antigens A and B are more stable than protein antigens, and resist drying, heat, and the action of a number of drastic chemical reagents. Since they can be demonstrated rather readily (by skilled technicians) in dried blood, it was natural to test the possibility that it might be possible to determine the blood groups of ancient human remains, such as mummies (11). This has apparently proved pos-

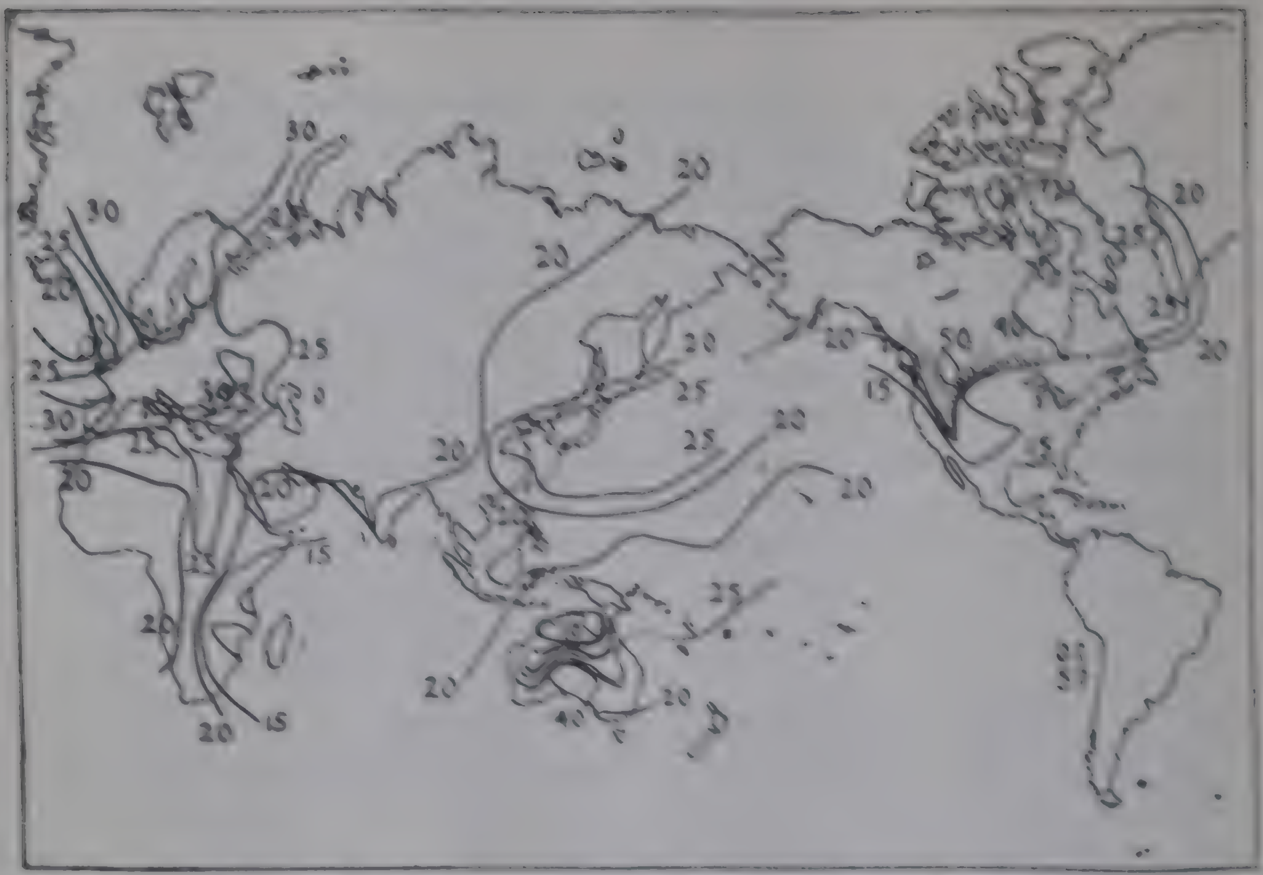

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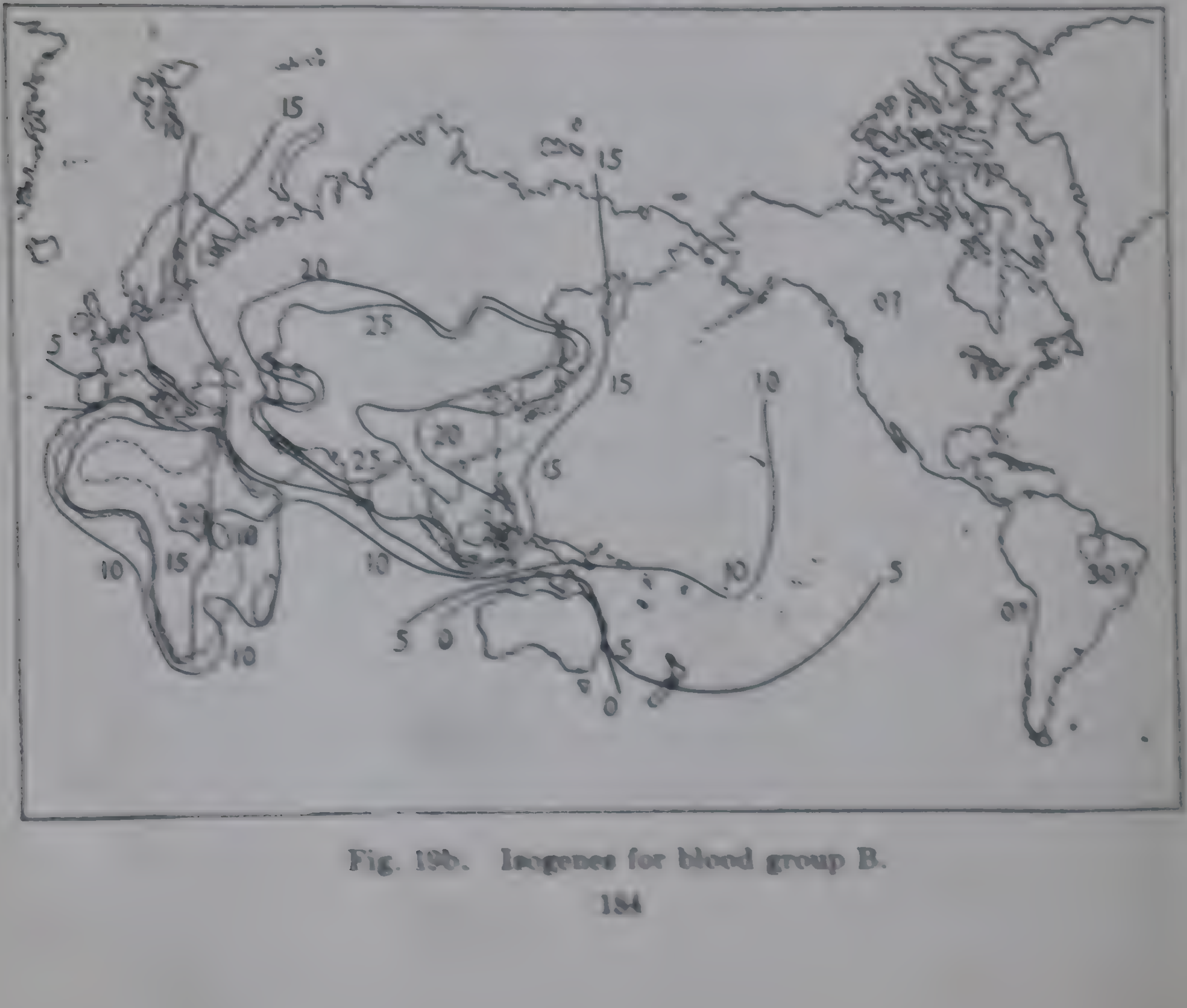
Fig. 19a. Isogenes (lines connecting points where same gene frequency is found) for blood group A in the world.


15
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5
0
5
0
0
30

Fig. 19b. Isogenes for blood group B.

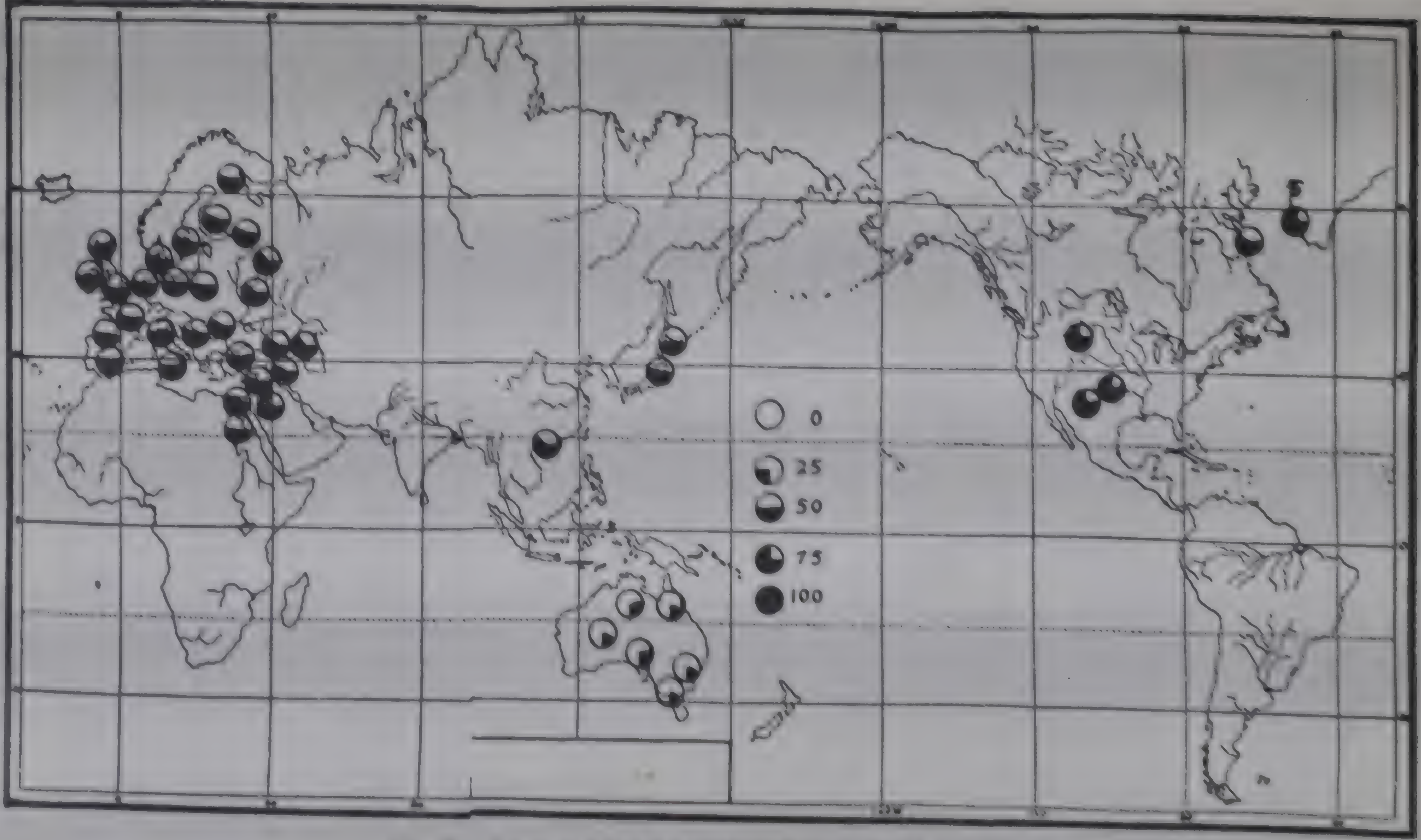

0
25
50
75
100

Fig. 20. Frequency of M gene in various parts of the world

sible, and over 300 mummies have been tested, with results in general conformity with what would have been expected. Work by Candela indicates that this technic can be applied also to ancient bone (see 76).

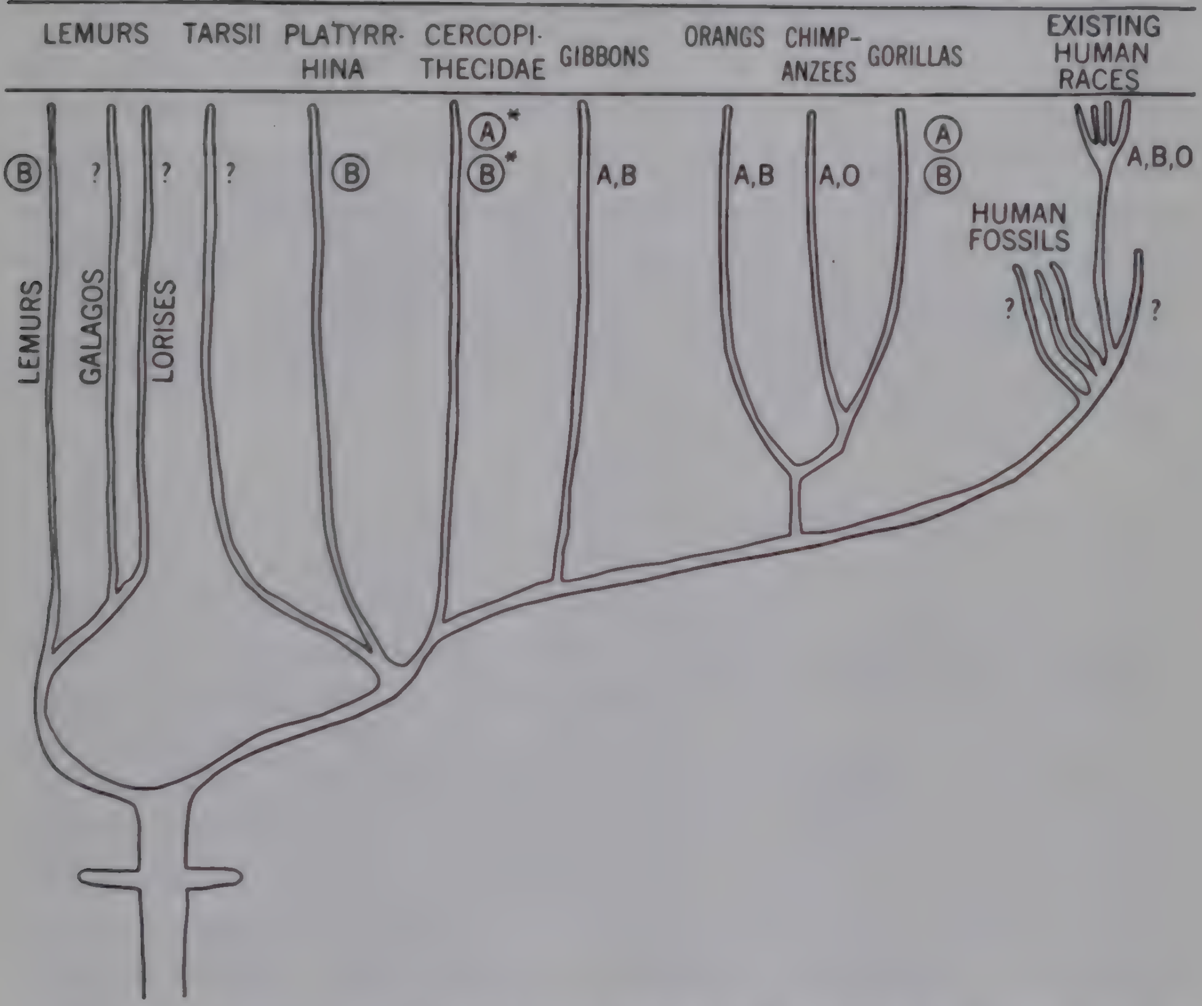
17. Related Factors in Animals

Antigens similar to the A and B in man occur in some animals (see 31). Such antigens are found, for example, in the saliva of the horse. In this animal A and B, together, alone, or neither, may occur, thus establishing four groups, probably hereditary, similar to those in man; but the antigens are not present also in the blood, as they are in man (30). In other animals the erythrocytes contain similar, but distinct, antigens. Certain workers have separated A and B into various hypothetical fractions (see 4) by comparing them with such related antigens. To this hypothetical fractionation the same objection is possible as in the case of related bacterial antigens (see Chapter IV), although in some cases it may be justified.

The greatest interest for students of human evolution attaches of course to the question of blood group factors in the apes and monkeys, and considerable work has been done on this line (for instance, 46). It has been found that in the higher primates substances indistinguishable from the A and B of man may be found; in the gorilla (16) and the monkeys a substance reacting with the anti-B agglutinin, but somewhat different from B, is found. See Figure 21.

From this it may be concluded that properties related to A and B are by no means restricted to mankind, but are widely distributed through the animal kingdom. There is a striking analogy with the Forssman hapten and with the specific carbohydrates of certain bacteria. Since there is reason to think (see Chapter III) that the possible chemical variety in the carbohydrate series (to which these antigens all belong, at least in part) is less than in the protein series, this may account for the more marked interspecific reactions observed also in the present instance. The probability of two carbohydrates taken at random being serologically similar would be greater in general than that for two unrelated proteins. A carbohydrate which is capable of reacting with either anti-A or anti-B has been isolated from *Ascaris* (69) and other helminths (70).

It has been found that the higher primates also possess blood structures capable of reacting with antisera for M, but the degree of reaction is often quite different with blood from the same animal when different anti-M sera are used. It does not seem that the M factor is ever lacking in any individuals of a primate species in which it is found, indicating a difference in the genetic mechanism from that operating in man (47, 80).


LEMURS
TARSII
PLATYRRHINA
CERCOPITHECIDAE
GIBBONS
ORANGS
CHIMPANZEES
GORILLAS
EXISTING HUMAN RACES
Ⓑ
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Ⓑ
Ⓐ*
Ⓑ*
A,B
A,B
A,O
Ⓐ
Ⓑ
A,B,O
HUMAN FOSSILS
?
?
LEMURS
GALAGOS
LORISES

Fig. 21. Occurrence of blood factors O, A, and B in man and other primates (after Landsteiner). Encircled A and B indicate A-like and B-like antigens reacting with human anti-A and anti-B agglutinins. Encircled A and B with asterisk indicate A-like and B-like antigens found in the tissues and secretions.

18. Function of the Group Substances

It is tempting to speculate on the possible physiological function of the blood group substances,.especially in connection with the possibility that natural selection may have influenced their frequency in certain parts of the world. In particular the serological relationship of A to some bacterial carbohydrates suggests the (entirely unproven) idea that there might have been some selective action due to slight differential mortality to certain bacterial infections. But as yet absolutely nothing is known of any normal physiological function of the group substances, and we have no reason to think the members of any one group or type enjoy any superiority over their fellows. (The Rh type may constitute an exception.) The question is one of extreme difficulty, and it is hard to devise even a hypothetical experiment which would satisfactorily answer it.

19. SUBGROUPS

It was early found that there were two kinds of individuals of group A. The cells of one reacted strongly with anti-A agglutinin, those of the other (the less common) reacted weakly with anti-A (44). These have been designated as A_1 and A_2, respectively. The corresponding difference is observed in group AB, so that these two groups are subdivided into subgroups as follows:

$$A = A_1 \text{ and } A_2$$

$$AB = A_1B \text{ and } A_2B$$

The difference in reactive power of the two kinds of A may not always be striking, but it can usually be demonstrated by absorbing a serum of group B (containing anti-A) with cells of subgroup A_2 until the serum no longer acts on these cells. It will then generally be found that the absorbed serum will agglutinate only cells of groups A_1 and A_1B. However, cells of group A_2 are not completely devoid of absorbing action on the anti-A_1 agglutinins in the B serum. If larger amounts are used, effects on the agglutinating power of the serum for A_1 cells can be produced. The difference between the two types of cells is probably not purely a matter of quantity of A substance, however, as indicated by certain observations of Landsteiner and Witt (49) and Wiener (87).

It has also been found that A_2 cells react more strongly than do A_1 cells with certain sera agglutinating cells of group O (such as certain absorbed cow sera, goat anti-Shiga sera, etc.), and with certain weak agglutinins occasionally found in the serum of group A_1B or A_1. There have been a number of hypotheses (summarized by Boyd, 4) to account for the difference between these two forms of A.

The subgroups of A and AB seem to be inherited (44), although considerable variation in reactivity of the cells of various individuals belonging to the same subgroup has been observed (9). A hypothetical mechanism of inheritance, which is a simple extension of the

Bernstein theory for A and B, has been offered by Thomsen, Friedenreich, and Worsaae. The families thus far studied conform to this theory on the whole, but there have been a few exceptions.

A_3 and A_4

Fischer and Hahn (23) and Friedenreich (26) reported the existence of a third subgroup of group A, which was designated as A_3. It is agglutinated by B sera even more weakly than is A_2. It seems to be very rare (perhaps one out of 2000 persons). Gammelgaard and Marcussen (32) have reported the existence of still a fourth allelomorphic gene of A, designated as A_4. It is agglutinated even less readily than A_3; immune rabbit sera and certain O sera were found best for its detection. One example was found in 60,000 routine examinations.

N_2 and M_2

More weakly agglutinable forms of the agglutinogen N when combined with the M factor to form the heterozygote MN have been reported (see 27). They are of rare occurrence. One case of an M factor reacting more weakly with about half the anti-M sera, but more strongly with about one-fourth of them, has been reported (29).

20. OTHER AGGLUTINABLE FACTORS IN HUMAN BLOOD

The agglutinogens already discussed do not comprise all the factors differentiating human bloods. There are a number of others, and by utilizing all of them it would be possible to differentiate over 1000 different kinds of human blood. Most of the remaining factors, however, are all either too rare to be of great forensic importance, or the reactions obtained with them are too weak and variable to make their routine investigations easy. In addition to A, B, M, and N, factors called P, G, H, X, Q, and E, have been reported (4, 7). Other workers, such as Hooker and Anderson (37), Zacho (9[illegible]), Culbertson and Ratcliffe (17), Neter (68), Levine and Stetson (61), observed, but did not name, agglutinins and agglutinogens which seemed to fall outside the A, B and M, N schemes. The case of Levine and Stetson was particularly well described and (see page 191) later tests showed it to depend on the Rh factor.

A natural outgrowth of the new knowledge of blood incompatibility was the concept that transfusions of blood containing a factor not present in the recipient's serum, even though no natural isoagglutinin was present at the outset, might result in the formation of an immune isoagglutinin, or produce some other form of sensitization, so that later transfusions of blood containing this factor would eventually be harmful. This was specifically suggested in the case of the M, N series by Lattes (50), and the warning was reiterated by numerous others. Actually, the M and N factors, though antigenic for rabbits, seem seldom to be isoantigenic in man, so the difficulty which was feared did not immediately arise. In all the bloods that have been examined, only about six or seven cases of human anti-M (71, 86) agglutinins have been described.

Nevertheless, accidents and untoward results (19, 62) did continue to occur in transfusions much more frequently than was desirable, or than many clinicians were willing to admit. Some of them, but probably only a few, may have been attributable to the use of a "dangerous universal donor" (1, 18, 60, 67, 87), whose anti-A and anti-B agglutinins were unusually potent and, when transfused, caused destruction of the recipient's erythrocytes, or other undesirable effects.

21. Discovery of Rh

In 1940, Landsteiner and Wiener (48), using the serum of a rabbit immunized with the erythrocytes of the *rhesus* monkey, obtained an agglutinin which agglutinated the cells of about 85% of the white population of New York City, irrespective of the blood group. They designated the new agglutinogen, in conformity with the origin of the serum, as Rh. The technic of testing for the new factor was difficult, the best available sera were weak, and had it not been for a remarkable series of discoveries which followed in the next few months, the Rh factor might not have aroused much interest.

22. Isoimmunization of Mother by Fetus

To explain the occurrence of an atypical agglutinin in the maternal circulation in a transfusion case, Levine and Stetson (61) suggested the important idea that foreign antigen from the fetus (which was supposed to have inherited the factor from the father) passed through the placenta and, acting as an isoantigen, immunized the mother. The observations of Wiener and Peters (90), by demonstrating that the new Rh factor when transfused into Rh-negative recipients could act as an isoantigen, served to suggest that this idea was correct.

The ground was now all prepared, and the seed planted by Landsteiner and Wiener's discovery and characterization of the Rh factor produced a rapid and luxuriant growth of fruitful investigations. Wiener and Peters reported three hemolytic transfusion reactions due to Rh incompatibility (90). They suspected (54) that the transfusion reactions observed by Levine and Stetson and other previous workers were due to Rh incompatibility, and later when anti-Rh sera became available to Levine and Katzin (55, 58), these latter workers were able to show that such was the case: the patient was Rh-negative, the husband Rh-positive, the atypical agglutinin was anti-Rh in specificity, and all of the mother's compatible donors were Rh-negative.

Some human sera seem to contain an "incomplete" (73) or "blocking antibody" (85) for Rh, which prevents the action of anti-Rh antibodies, but does not itself agglutinate. Similar inhibiting antibodies have been reported for other agglutinating systems, e.g., Levine and Gilmore (57).

23. ROLE OF Rh IN ERYTHROBLASTOSIS

When Diamond, Blackfan, and Baty (20) suggested in 1932 that universal edema of the fetus, *icterus gravis neonatorum*, and anemia of the newborn were all manifestations of the same disease, and could conveniently be grouped together and known as *erythroblastosis fetalis*, no thought of a possible serological origin of the condition, apparently, was in their minds.

Following up the suggestion of Levine and Stetson (61), Levine *et al.* (56) considered more fully the role of isoimmunization in the pathogenesis of *erythroblastosis fetalis*. The latter authors considered that isoimmunization of an Rh-negative mother to the Rh antigen contained in her Rh-positive fetus, with subsequent passage of the immune anti-Rh agglutinin back across the placenta, was the cause of erythroblastosis in the great majority of cases. As statistical evidence they pointed out that whereas only 15% of the random population was Rh-negative, of the 153 mothers whose infants were affected with *erythroblastosis fetalis*, 141 (92%) were Rh-negative. Also, of 76 infants and 89 husbands tested in this group, *all* were Rh-positive. In 70 cases the mother's serum was tested for agglutinins within two months of delivery; anti-Rh antibodies were found in 33 of these. Confirmatory observations have been published (3a, 83).

Levine (53) has also suggested that serological incompatibility (perhaps in regard to other blood factors also) may sometimes be responsible for spontaneous abortions and still births. He (51) also mentions that several workers have reported a higher than normal incidence of congenital malformations in erythroblastotic infants.

It was clear from the start that the combination Rh-negative mother and Rh-positive fetus, which occurs in about 9.5% of all pregnancies in England and North America, does not automatically mean erythroblastosis, for the highest estimate of the incidence of this disease is only about 1 in 200 or 1 in 400 births (39a). As Mollison (64) points out, therefore, only about once in 40 times does the potentially dangerous combination actually lead to morbidity. Evidently certain other conditions must also be fulfilled. We do not know what they all are, but one essential seems to be the passage of Rh-containing fetal cells (or possibly just Rh antigen?) through the placenta into the maternal circulation, with resulting immunization (unless this has already happened in a previous pregnancy).

24. SUBDIVISIONS OF THE Rh TYPE

Soon after the importance of the Rh factor in *erythroblastosis fetalis* and in transfusion accidents was established, it was observed that various human anti-Rh sera did not exhibit identical specificities (56, 81). For a time the three principal types of such sera were designated by the characteristic percentages (56) of positive reactions obtained with them on bloods of the white population of New York and vicinity, that is, as 87%, 85%, and 73%, respectively. Levine (51, 52) showed that the 87% serum contained more than one antibody, and in fact it is now known that it contains in one serum the two agglutinins found separately in the 85 and 73% sera. At first it was thought that Rh could be separated simply into Rh_1 and Rh_2, as in the case of A (82), but later work (73, 84, 88) has shown that the subject is actually more complex than it first appeared. Therefore the earlier nomenclatures proposed for these agglutinins and their corresponding agglutinogens have been somewhat modified in the light of later results (91).

The subject is still developing so rapidly, and there is still so much doubt as to the genetic mechanisms involved, and the proper gene symbols to use, that the reader desiring to know the present status of the subject will have to consult the current periodical literature.

According to Wiener's nomenclature, the allelomorphic genes Rh_o, Rh', Rh'', $Rh_1 (= Rh_o')$, Rh_2 $(= Rh_o'')$, Rh_y, Rh_z, and rh (= Rh negative), must be recognized. The English workers have proposed a totally different system of nomenclature, postulating three closely linked gene loci C, D, and E. The genetic predictions of the two systems are nearly the same, but the philosophy underlying them differs profoundly. A good account of the state of the subject at the moment of correcting proof will be found in two recent reviews (55a, 85a).

SUMMARY

Ca. 1900 A.D. individual antigenic blood differences, often as great as those which separate species, were found to exist in certain species, particularly man. (*1*) Two blood group substances, A and B, are found in human erythrocytes and other cells. Related substances are found in lower animals. Gastric juice and stomach are among the best sources for the substances, which when purified are found to be nitrogen-containing carbohydrates. There is probably also an O substance, related to, but different from A and B. (*2*) The four classical human blood groups depend upon the presence or absence of the A and B substances (agglutinogens) in the erythrocytes. Four combinations are possible; O, A, B, and AB. (*3*) In the blood serum agglutinins for A, or B, substances not present in the erythrocytes are found (Landsteiner's rule). Such agglutinins may be produced artificially in animals, and those present in man may often be increased in strength by injection of A substance (hog) and B (+ A) substance (horse). By using anti-A and anti-B agglutinins, the four groups of human blood are diagnosed. (*4*) Blood transfusions were seldom successful before the discovery of the blood groups, since blood of a different group may be incompatible. The main source of reaction is action of the agglutinins of the recipient on the donor's cells. It is well to consider the converse reaction, however, unless the emergency is great, as in war time. (*5*) The four classical blood groups are inherited by means of a series of allelomorphic genes, O, A, B. Since each individual can have only two of these genes, and since the heterozygotes AO and BO are indistinguishable routinely from AA and BB respectively, this gives the four classical types, and if we know the genetic constitution of the partners of a mating, we may predict to what blood groups their children may belong. (*6*) Such genetic knowledge enables us to apply blood groups to questions of disputed parentage, and even to compute probabilities of excluding an individual as parent. Other forensic applications include the determination of the blood group of blood stains or stains made by semen, saliva, etc. (*7*) The group substances occur outside of the erythrocytes, in most of the cells and tissue fluids. (*8*) Enzymes exist which have the power of destroying the specific activity of the blood group substances. (*9*) Presence or absence of the blood group substances in the saliva or gastric juice, or in the tissues in water-soluble form, seems to depend on the presence or absence of another gene (S). (*10*) Two additional antigens, M and N, in human erythrocytes were discovered by Landsteiner and Levine. (*11*) M and N must be detected by the use of immune serum from animals. (*12*) They are inherited as a simple Mendelian pair of genes without dominance. Three types thus result, independent of the A, B, O groups. (*13*) These factors can also be used in cases of disputed parentage. (*14*) All these blood factors seem to be present at birth in those individuals who inherit them, and even to develop during fetal life. The agglutinins may not develop until later. (*15*) The frequencies of the different groups and types is different in various parts of the world. They do not enable "races" to be distinguished, but do provide valuable objective methods of classifying mankind. The gene frequencies are generally used for this. (*16*) The blood group substances are so stable that the blood group of human remains, including mummies, may often be determined. (*17*) Factors related to A and B occur in animals, and those in the other primates are very similar. (*18*) The physiological function of the group substances, if any, is unknown. (*19*) Groups A and AB may be subdivided into subgroups, $A_1 + A_2$ and $A_1B + A_2B$. The A_2 factor reacts more weakly than A_1 with most anti-A sera. Other subgroups have been reported. (*20*) Still other agglutinable factors have been found in human blood. (*21*) Of these, the most important clinically seems to be the Rh factor. (*22*) Rh incompatibility explains many cases of a rare disease of infants, *erythroblastosis fetalis*, and also many transfusion reactions in adults. Agglutinins for Rh do not occur naturally, but may arise as the result of stimulation. An example of such stimulation is provided by the case where an Rh-negative mother becomes isoimmunized to Rh from the erythrocytes of an Rh-positive fetus she is carrying. Rh also may act as an isoantigen when Rh-positive blood is transfused into Rh-negative recipients. Agglutinins for the Rh factor can often be demonstrated in cases of isoimmunization, but an "incomplete" or "blocking" type of antibody, not detectable without special measures may be found. (*23*) It is thought that anti-Rh agglutinins produced by the (Rh-negative) mother of an Rh-positive fetus diffuse back through the placenta into the fetal circulation and are responsible for the hemolytic and other damage which is characteristic of hemolytic disease of the newborn. *(24)* The Rh factor is antigenically and genetically complex, and at least six genetic factors must be assumed to explain the inheritance of all the types observed.

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Chapter V

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CHAPTER VI

ANTIBODY–ANTIGEN REACTIONS

The majority of those actively engaged in studying immunological reactions are convinced that the reaction between antibody and antigen is chemical in nature. Certain difficulties have arisen in the attempt to interpret all the observations in a consistent way, possibly because of the size and complexity of the molecules involved. The reaction between antibody and antigen, and that between proteins and proteolytic enzymes, are the only instances which have been well studied of reactions occurring between molecules of very large size; it need not be surprising if some new features emerge. Reserving for later discussion the reaction with haptens, which are usually much smaller molecules, and the combination of antibody with intact cells such as bacteria or erythrocytes, what do we find to be the salient characteristics of the reaction? There are several features which stand out.

1. Basic Facts

(*a*) *The reaction is specific.* This point has already been extensively discussed, and it will not be necessary to give any examples here. We shall have to keep in mind the specificity of the reaction when we come to discuss the mechanism of union, that is, the nature of the forces holding antibody and antigen together.

(*b*) The *entire* molecules react, not some fragment split off from them. This is clearly shown by ultracentrifugal experiments (see 86, 146).

(*c*) The antibody molecule, and molecules of protein antigens, at any rate, behave in these reactions as fairly *rigid* ellipsoids of greater or less eccentricity. This is obvious if we accept the views of Neurath (141) on the shape of protein molecules. Boyd and Hooker (24, 27) found that the composition of antibody–antigen precipitates is compatible with the idea that antigen molecules are roughly spherical and antibody molecules behave as elongated units, such as ellipsoids, or chains of connected spheres. It is also found in antibody–antigen reactions between films of the reagents (e.g., see 160) that the thickness (30–50 Å.) of the films is such as to suggest that the molecules are behaving like ellipsoids lying on their sides. Evidence that protein molecules act as fairly rigid spheres or ellipsoids comes from their behavior in intense gravitational fields (169), and their possession of a definite electric moment (184). See also (134, 182).

Certain earlier workers apparently supposed that the antibody spread as a thin film over the surface of the antigen. Such a film would resemble a condensed protein film, and having the polar groups turned away from the surrounding water, could account for the hydrophobic character of the compound.

The above evidence, however, indicates that antibody does not behave as such a film. Also, the amount of antibody observed to combine with a molecule of antigen does not correspond to a film approximately 10 Å. thick as this could lead us to suppose, but to a layer more than three times as thick (see page 238). Also, the electrokinetic properties of the antibody–antigen complex are still those of protein, and do not suggest that all the polar groups are turned away from the surface.

(*d*) *No splitting, digestion*, or other profound chemical alteration takes place in the reacting molecules after they have combined.

There are numerous observations showing that no profound alteration takes place in the antigen and antibody molecules after they combine. It is a well-known fact that toxin is not permanently detoxified, but simply neutralized, by combination with antitoxin. Severe reactions resulted in two communities following the injection of toxin–antitoxin mixtures (of a formula not used at present) which had been frozen, apparently with resulting damage to the antitoxin and consequent release of some of the toxic properties of the toxin (162). Venoms can be recovered after neutralization with antivenins, by destroying the antibody (33, 135).

Similarly, various workers (see Chapter II) have been able to recover unaltered antibody from antibody–antigen precipitates or "agglutinates." It is evident that if any alteration takes place, it is pretty much reversible (35). It has been thought by a number of workers that the antibody is denatured after combination with antigen. Probably without fully sharing this idea, Eagle (53) and Mudd (see 138) emphasized the similarity in properties of combined antibody and denatured globulin. This similarity certainly exists, and is probably significant for theories of serological reactions, but it need not mean that the antibody is actually denatured. Eagle suggests that it is due to the (assumed) turning of the polar groups of the antibody towards the polar groups of the antigen, leaving a surface composed predominantly of nonpolar groups, with a consequent reduction of solubility in polar solvents such as water and saline.

Completely denatured proteins are often found to have reverted to the fibrous form, which may be detected by X-ray analysis; therefore Marrack (129) believes that if the antibody in specific precipitates were completely denatured this should be detectable by X-ray study, but actually experiments have not produced any evidence that the antibody in such precipitates has changed from the globular form. We may therefore conclude that the antibody in such precipitates is not denatured, at least not wholly.

Not only is the antibody not immediately denatured, but it has been found (16) that the solubility of ovalbumin–antiovalbumin precipitates in excess of antigen was not altered by storage (see page 215). Since this is a specific reaction, the result indicated that no progressive loss of specific reactivity of either of the reagents had taken place during that time.

There is other evidence that the serological specificity of the antigen and antibody is not altered by their combination. Precipitates will specifically sensitize animals to the antigen, and to the serum proteins of the animal from which the antibody was derived; nonprecipitating (inhibition zone) compounds of antibody and antigen can be precipitated, after the lapse of an indefinite time, by the addition of more of the deficient reagent, or by the addition of a different antibody directed against either the antigen or the first antibody. This again indicates that no significant denaturation takes place.

(*e*) The combination of antibody and antigen takes place at the *surface* of the molecules. This follows from the third point discussed above. Also, Marrack (129) has pointed out that no molecule larger than the lower fatty acids could pass between the constituent atoms of a protein molecule, in view of their known distances from each other. Evidence for the role of surface in serological reactions has been brought out in the preceding chapters; it has been stressed by Hooker (98). The experiments of Marrack suggest that when the antibody molecule is unfolded, thus producing a new "surface," it loses its power of specific combination, and the experiments of Porter and Pappenheimer (160) indicate that the reactive capacity of the antitoxin molecule is restricted to a portion of its surface.

It has been suggested (8, 12) that the protective action of antipneumococcus antibodies, for instance, is due to their combination with the surface of the bacteria, and Bjørneboe (12) suggests that the greatest effect is obtained when the antibodies are evenly distributed over the surface.

(*f*) The union between antibody and antigen is a *firm one*, but is at least partially *reversible*. It is well known that it is quite difficult to dissociate diphtheria antitoxin and toxin after their combination (see 146). It is possible to dissolve the precipitate formed by the conjugated protein, arsanilic-acid-azocasein, and its antibody by the addition of dilute alkali; addition of dilute acid to this solution, which would not precipitate the antibody alone, throws down the antibody and antigen together, with hardly a trace of antibody left in the supernatant fluid (15). Studies on the heat of serological reactions (23) indicate a considerable energy change, implying a rather firm combination.

Nevertheless, the reversibility of serological reactions is shown by several lines of evidence, such as dissociation of antibody from precipitates and agglutinated cells, and solution of precipitates in excess of antigen. This evidence is presented more fully on page 215.

(*g*) *Both antibody and antigen enter into the specific precipitate*, or into the specific "agglutinate." There is now abundant evidence for this statement, but before the introduction of quantitative methods into immunology the question was sometimes debated.

(*h*) *Antibody and antigen can combine in varying proportions.* This is an obvious consequence of the multivalency of one or the other of the reagents, and has been shown by numerous analyses. In the light of what we know now, it may seem surprising that it was ever doubted, yet it is precisely the failure to recognize the possibility of combination in different proportions which caused the early writers on antibody-antigen reactions so much difficulty. However, once it was realized that either antigen or antibody, or possibly both, had to be multivalent, the observation that antibody-antigen compounds may vary in composition becomes quite understandable. Credit for insisting on this point, and providing evidence for it, must go largely to Heidelberger and collaborators.

(*i*) Serological reactions display certain analogies with colloidal reactions. (See page 207).

2. The Two Stages of Serological Reactions

It is customary to consider serological reactions as occurring in two stages. Such a subdivision should not be taken as necessarily implying that the two stages do not overlap, for it is quite likely that the second stage sets in before the first is entirely completed.

These two stages are: the specific combination between specific groups of the antibody and corresponding groups of the antigen or hapten; and the secondary, observable reactions which may follow this, such as precipitation, agglutination, and complement fixation.

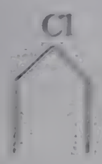
There are several features of these reactions which make it convenient for us to retain this division into stages. (*a*) The first stage proceeds without visible alteration, and can be detected only indirectly, while the second may be quite conspicuous and easily detected, (*b*) in the case of small haptens, as a rule, no second stage follows at all, (*c*) under some conditions, as in the absence of salts, the first stage can take place, but the second cannot, (*d*) their speeds are very unequal, the first stage being extremely rapid, and the second sometimes very slow, (*e*) the energy change seems to take place during the first stage, the second being accompanied by little energy change, (*f*) there is some evidence (see below) that the specificity of the second stage may be of a lower order than that of the first stage.

A. First Stage: Combination

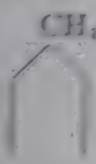
1. MODE OF COMBINATION BETWEEN ANTIBODY AND ANTIGEN

Because of the chemical nature of serological specificity, it is logical to suppose that the union between antibody and antigen is a chemical one, due to combination of the specific reactive groups of the two reagents. This is supported by such facts as the power of specific simple chemical substances (haptens) to react with antibodies, and by the analogy with enzyme reactions, known to be chemical in nature.

For a long time the principal question in this connection was: is the union solely due to coulomb forces (attraction between positive and negative charges, e.g., $-NH_3^+$ and $-COO^-$) or are other forces involved? If the forces acting are coulomb forces, we should suppose that the combination between antibody and antigen is chiefly due to attraction between amino and carboxyl groups. The possibility of such an ionic mechanism was pointed out by Heidelberger and Kendall in 1929 (78). There are points in support of this idea, such as the rapidity of the primary reaction, and the strong influence of polar groups observed in studies of specificity. In particular the immunological equivalence of such groups as



and



and the acidic character of serologically reactive nonprotein antigens, such as some of the pneumococcal polysaccharides, are suggestive. Goebel and Hotchkiss (70) found that antipneumococcus horse sera of types 1, 3, and 8 gave vigorous precipitation with artificial antigens containing benzene carboxylic and sulfonic acid radicals, which are quite unrelated in chemical constitution to the pneumococcus polysaccharides. Chow and Goebel (34) suggested that combination between the pneumococcus polysaccharides and the corresponding antibodies might largely be due to attraction between the ionized uronic acid groups, containing $—COO^-$, and the ionized amino groups, $—NH_3^+$, of the protein. In support of this idea, it was found that treatment of the antibody with formalin, which reacts with the amino groups, destroys the precipitating power of the antibody, and that acetylation, also affecting the amino groups, is almost equally effective.

Landsteiner (119), in favor of the idea that such a mechanism, which he refers to as the formation of salt-like compounds, may operate, lists the following: the prominent influence of acid groups (see page 103), the change in specificity following esterification of aromatic acids or proteins, and the similarity of hemagglutination and hemolysis produced by serological agents and by colloidal inorganic acids.

It has been objected that such a simple mechanism could not account for the specificity of serological reactions, but this is perhaps not a necessary difficulty. If a number of amino and carboxyl groups in the antibody together form one specific determinant group for a determinant group of the antigen, the specificity might be due to the spatial arrangement of these groups. The weaker union observed with related heterologous antigens could be explained by a failure of the oppositely charged groups to correspond perfectly in position.

It is however likely that other forces, such as those classified as secondary valence, play a role in serological reactions (151, 153). It does not seem likely that the covalent bond is concerned, for, as Landsteiner says (119): ". . . in velocity and easy reversibility antigen–antibody reactions differ from those due to primary valences and resemble the formation of ionic and molecular compounds; and a strong argument against the assumption of covalent bonds is the fact that quite different substances, regardless of their chemical nature, are capable of reacting with antibodies all in like manner." It is likely that similar objections apply against the co-ordinate link, or semipolar double bond (see 149). Other types of bonds which may possibly play some role in serological reactions are dipole–dipole bonds, dipole–ion bonds (shown schematically in Fig. 22, see 71), van der Waal's forces, and the hydrogen bond (151, 154). The latter consists essentially of a hydrogen atom which is attracted simultaneously to two different atoms, as in salicylaldehyde.

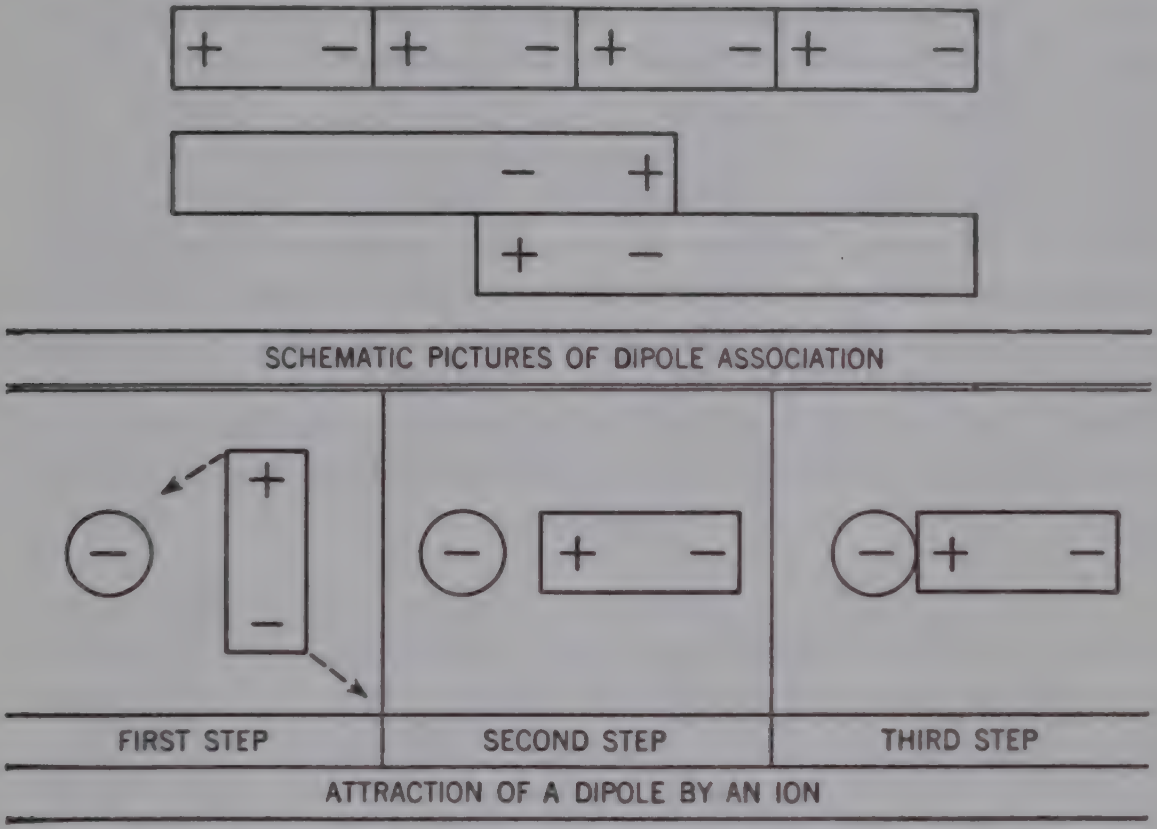
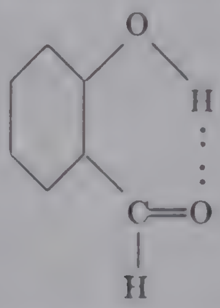

SCHEMATIC PICTURES OF DIPOLE ASSOCIATION
FIRST STEP
SECOND STEP
THIRD STEP
ATTRACTION OF A DIPOLE BY AN ION

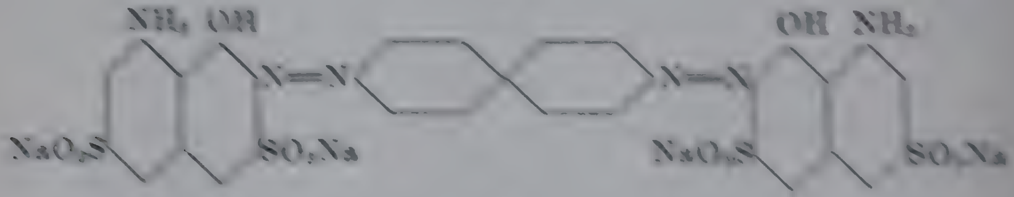
Fig. 22. Possible ways in which forces between dipoles and dipoles and ions might enter into the reaction between antibody and antigen (71).



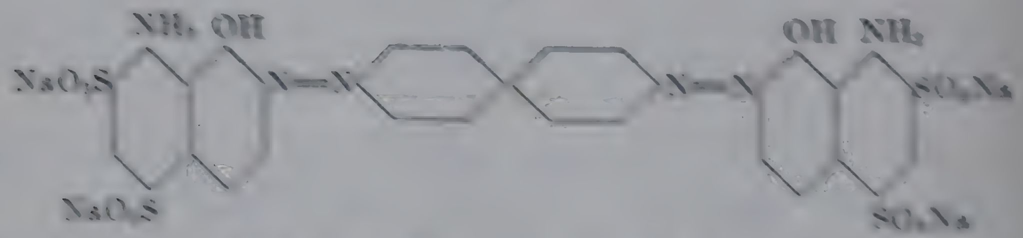
The hydrogen bond, which has been discussed, for example, by Pauling (149) appears to be one of the forces keeping proteins in their characteristic configurations.

Other writers have mentioned cases of chemical specificity which compare in this respect with serological reactions. Thus Landsteiner (119) calls attention to the very interesting examples of the reactions of copper with the $\mathrm{HO}\overset{|\ |}{\underset{|}{\mathrm{CC}}}\mathrm{:NOH}$ group and thalium with $-COCH_2CO-$, and the observations of Bergmann on the precipitation of proline and some peptides by rhodanilic acid, and the distinctly specific precipitation of glycine, the simplest amino acid, with potassium trioxalatochromiate, $Cr(C_2O_4)_3K_3 \cdot 3H_2O$. Marrack (129) has drawn attention to the analogy with certain cases of remarkably specific adsorption to crystal surfaces. Thus methylene blue is adsorbed by diamond and not by graphite, succinic acid by graphite and not by diamond. The only difference between these two crystals is the spacing of the carbon atoms; it will be recalled that we have already found spatial arrangement to be highly important for specificity. The dye I (below) is adsorbed on the crystal faces of potash alum, while its isomer, II, is not. Differences have even been found

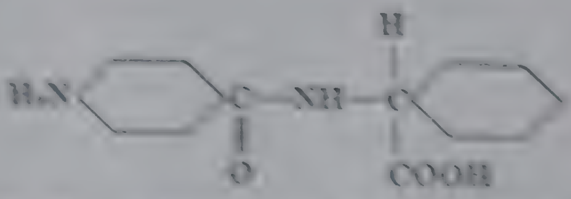
I



II



in the degree to which optical isomers are adsorbed. Thus the dextro and levo forms of the dye made from phenyl-(p-aminobenzoyl)-aminoacetic acid



and dimethyl aniline have proved to be adsorbed unequally by wool. It is certainly by no means inconceivable that the reaction between antibody and antigen may be at least in part mediated by the same forces which act in these cases. These forces however, though different in kind, are probably no less chemical than the forces holding sodium and chloride ions together in a crystal of sodium chloride. As Langmuir (125) puts it, there is "no fundamental distinction between chemical and physical forces."

2. Analogies with Colloidal Reactions

Serological reactions have a number of points of similarity with many colloidal reactions, as was well brought out by Bordet (13). A consideration of these analogies will be helpful in understanding antibody–antigen reactions.

One important similarity is the ability of the reagents to enter into composition in almost any number of varying proportions, depending upon the ratio in which they are brought together. This is in sharp contrast to the behavior of simple chemical agents, and is evidently in both cases a consequence of the large number of reactive groups possessed by one or both of the reagents. Thus in serological reactions we find, as in colloidal reactions, that, if an excess of one reagent is added, the resulting compound contains more of this reagent, but *not more in proportion to the excess added*. That is, as more reagent is added, the additional amount taken up begins to taper off, giving a curve which flattens out, like an adsorption isotherm. This is well shown by figures such as those in Tables XXXIII, XXXVI, and XXXVII on pages 242, 269, and 272, respectively.

The two sorts of reactions also resemble each other in the importance of electrolytes, which in each case have considerable influence on the rate of reaction and the stability of the reagents. In both cases it may be found that without electrolytes the reaction does not go, at least to the extent of producing a visible change. The electrolyte concentration is also sometimes observed to have an important effect on the composition of the product which is formed. We may suppose that the effect of electrolytes rests in both cases on the important role played by electrical charges in the stability and reactivity of the substances.

The slow or occasionally imperfect reversibility of the reaction is also characteristic. It is well known that time is required for attainment of many colloidal equilibria, and that, if an excess of one agent is first added, the supersaturated compound which is formed is often only slowly converted to a compound of more "normal" composition when a corresponding amount of the second reagent is added. This is extremely reminiscent of the Danysz phenomenon in toxin–antitoxin reactions, discussed on page 225. The effect of temperature, which may in some cases markedly diminish the amount of product or prevent its appearing, is also analogous.

Just as antibody and antigen when mixed often produce a precipitate, so colloids often precipitate each other. In this case the colloidal particles usually carry charges of opposite sign, which is generally not the case with antibody and antigen, but colloidal particles of the same sign may also combine (63). Most striking of all is the fact that in both cases there is usually an "optimal" proportion in which the two reagents precipitate each other most rapidly and completely; if an excess of one, or sometimes either, reagent is used, precipitation is diminished, or absent. A good illustration of this is seen in the reaction between ferric oxide sol and arsenious sulfide sol (Table XXXII).

TABLE XXXII

FLOCCULATION OF AN As_2S_3 SOL BY A Fe_2O_3 SOL (10)

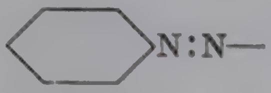
Mixture[a]		Degree of precipitation	Charge on particles in suspension
mg. Fe_2O_3	mg. As_2S_3		
0.61	20.3	Cloudy	−
6.08	16.6	Immediate, partial	−
9.12	14.5	Nearly complete	
15.2	10.4	Immediate, partial	+
24.3	4.14	Delayed clouding	+
27.4	2.07	None	

The Fe_2O_3 particles are positively charged; the As_2S_3 particles negatively.
[a] Total volume 10 cc.

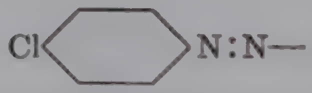
It will be noted that a well-defined optimum, quite similar to those observed in some serological reactions, is obtained. In this case it evidently occurs at the point where the charge on the particles resulting from union of the two oppositely charged colloids is at a minimum; the reduction in the electrical repulsive forces allows the particles to approach each other closely enough to stick together. Ways in which serological reactions may differ from this will be discussed later.

3. SIZE OF REACTING GROUPS

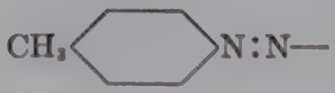
The size of the specific determinants in natural proteins is still not exactly known (see page 89), but in the case of artificial antigens we can form some idea of the extent of the reacting group. The total reacting group in conjugated antigens is probably somewhat larger than the introduced group which chiefly determines the specificity because of some influence contributed by adjacent groups of the native protein. The specificity of proteins can be altered by introducing the phenylazo group:



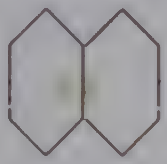
into them by treating them with diazotized aniline, but antigens containing this group differ but little from those containing chlorophenylazo:



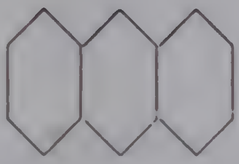
and methylphenylazo:



(see page 104). It was found that the reaction with such an antigen was not inhibited by aniline itself, in contrast to the results obtained with haptens containing polar groups. Jacobs (110) found that although introduced naphthalene residues:

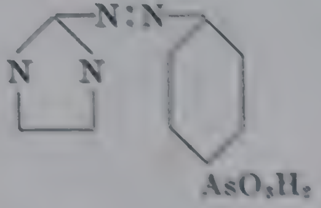


and anthracene residues:

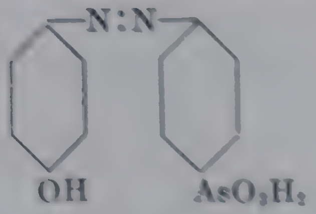


(both coupled through the azo (—N:N—) link) conferred a new specificity on the conjugated protein, the two antigens differed but little serologically in spite of the considerable structural difference between these haptens. These facts suggest that in such cases the groups of the antigen which enter into combination with the antibody are probably native groups, possibly altered somewhat in position, in the conjugated protein, lying near the introduced hapten. It is possible that the antibody reacts either not at all, or only to a limited extent with such "neutral" haptens alone. If the specific active groups of the antibody are directed, not towards the hapten alone, but also towards the tyrosine and histidine nuclei with which diazonium compounds couple in a conjugated protein, we should expect that such antisera would be inhibited most effectively, not by the hapten alone, but by the hapten coupled to tyrosine or histidine, or some other phenolic or imidazole compound; this has been observed by several workers.

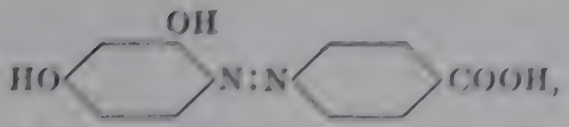
This idea is supported by the observation of Mutsaars and Grégoire (139) that synthetic antigens in which the determinant group (D) is coupled to the protein (Pr) through the lysine by a ureido link, DNHCOPr, are not equivalent to those in which it is attached by an azo link, DN:NPr. Hooker and Boyd (99) found similar evidence while studying antisera to egg-white–arsanilic-acid and gelatin–arsanilic-acid. Since gelatin contains no tyrosine, or but a negligible amount, it would be expected that the antibodies to this compound would have predominantly an arsanilic-acid–azohistidine specificity, while those to the egg white compound would be directed also partly towards tyrosine-like structures. Consistent with this prediction they considered their observations that the dye:



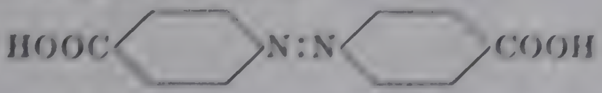
designated as H, was about three times as effective as another:



called T, in inhibiting the reaction of the anti-egg-white–arsanilic antibodies with casein–arsanilic, whereas it was ten times as effective with the anti-gelatin–arsanilic serum. The sodium salt of arsanilic acid alone (atoxyl) would also inhibit these sera, but much larger amounts were required. In later work Hooker and Boyd (105) found that antisera against *p*-aminobenzoic-acid-coupled antigens were inhibited much more efficiently by 2,4-dihydroxy-4′-carboxyazobenzene:

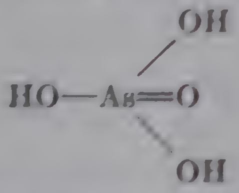


than by 4, 4′-dicarboxyazobenzene:

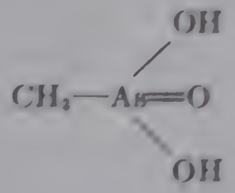


indicating presumably the influence of the greater similarity of the former to the structures beyond the azo group in the coupled protein. The above facts may be taken as indicating that the reactive groups in serology are of about the dimensions of a synthetic chemical compound like some of those represented here, or perhaps in some cases a bit larger.

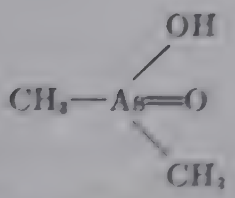
In some cases a simple subgroup may apparently determine the specificity to a large extent, as Landsteiner (118) and Haurowitz and Breinl (72) found that the inorganic arsenic acid:



had a weak inhibiting action on antisera to conjugated antigens containing arsanilic acid, $H_2NC_6H_4AsO_3H_2$. However, methylarsenic acid:



and cacodylic acid:



had no inhibiting action. Marrack (128) suggests that the inhibition observed with arsenic acid might have been nonspecific. In any case, arsanilic acid is found to be much more efficient as an inhibitor in such systems, and the azo dyes made from diazotized arsanilic acid are even more potent.

4. Nature of the Reactive Group

We may suppose, according to the above, that the specifically active "patches" on the surface of the antibody are adapted to react with a considerable area of the determinant group. The reaction is therefore likely to be a complex one, with several chemical groups which together form the "active patch" being involved, not all of them, perhaps, exerting the same sort of forces. The specificity conferred by a small introduced chemical group would be due partly to its own structure and partly to alteration in the neighboring structures in the protein molecule. Marrack (129) says: "The effect of changes such as the introduction of, for example, a Cl atom into the benzene ring of a determinant group may be ascribed both to a local effect on the electric field, affecting the local adaptation to the receptor site, and to an electron drift in the benzene ring, which affects the adaptation of the whole ring or of any dominant group attached to it, to the receptor site."

A purely schematic representation of how an extremely simple case of correspondence between the specific groups in the antibody and those in the antigen might be based on distribution of positive and negative groups, and hydrogen bonds, is shown in Figure 23. The H in this drawing may be taken to represent, either a hydrogen atom capable of taking part in hydrogen bond formation, or a site capable of attracting such a hydrogen.

If the antibody-combining groups are chemically complex, as supposed here, we shall not expect to find that antibody molecules always carry a positive charge, say, while antigen molecules are always negative, or anything so simple. Landsteiner (119) says: "While experiments by Michaelis and Davidsohn and de Kruif and Northrop contradict the 'idea that the combination (of antibody and antigen) is caused by opposite electric charges' they do not preclude that among the combining groups involved in the reactions, there are acid and basic groups interacting with each other." If we may provisionally accept this point of view, we should probably assume that acidic and basic groups enter in roughly equal numbers into the reaction, as most protein antigens are not notably either acidic or basic, and Smith and Marrack (166) found no *p*H change following an antibody–antigen reaction.

If any considerable part of the forces uniting antibody and antigen are coulomb forces, it might be expected than an antibody to a strongly acidic antigen, such as one of the pneumococcus polysaccharides, would have more free amino groups (and perhaps more imidazole and guanidino groups) than normal globulin, but it is not necessary to suppose that the increase would be readily detectable chemically. In the first place, if antibody contains only one or two reactive groups per molecule (see Chapter II), the change would be of the order of two amino groups out of some one hundred. Marrack (129) has calculated that even if antipneumococcus antibody contains enough extra amino groups to combine with all the carboxyl groups of the polysaccharide, this would involve an increase in the total amino content of the protein of only 14%. Rather precise analysis would therefore be required to detect the chemical differences which may exist between antibody and normal globulins.

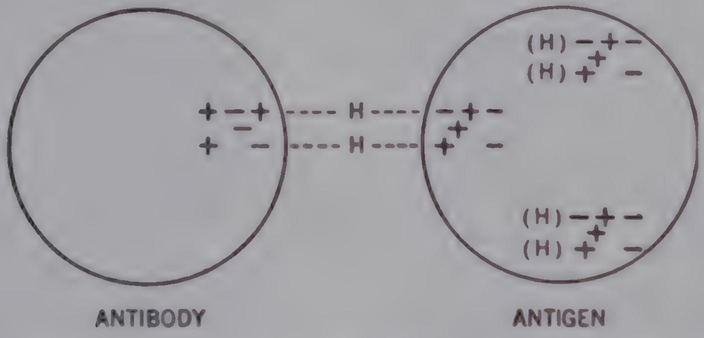


Fig. 23. Hypothetical way in which charges of opposite sign and groups capable of entering into hydrogen bond formation might determine the specific reaction between antibody and antigen.

There is no evidence that antibodies contain prosthetic specific reactive groups, and it is quite generally supposed that the reactive group of the antibody is simply a patch of the surface in which the various groups are arranged so as to correspond in some way, perhaps electrically, to those of the antigenic determinant. Haurowitz (71) supposes that the surface of the antibody corresponds to the surface of the antigen as an electrotype to an electrode of complicated shape. Pauling (150) supposes that a hapten or antigenic determinant fits into a pocket in the antibody and that the fit is a close one. This correspondence must be brought about, if these ideas are correct, by alterations of the spacing and arrangement of the amino acids in the antibody (see Fig. 10) while it is being synthesized.

Haurowitz (71) has also given a schematic representation of how the groups in the reactive portion of the antibody surface might be arranged to correspond to an artificial determinant, in this case metanilic acid. This is shown in Figure 24 (redrawn). It was of course realized by Haurowitz that any such picture is a gross oversimplification. We do not know enough of the structure of any antibody to attempt an accurate picture for an actual case. On the basis of pictures such as these, it is possible to understand how the spatial arrangement of the groups in the active patch of the antibody

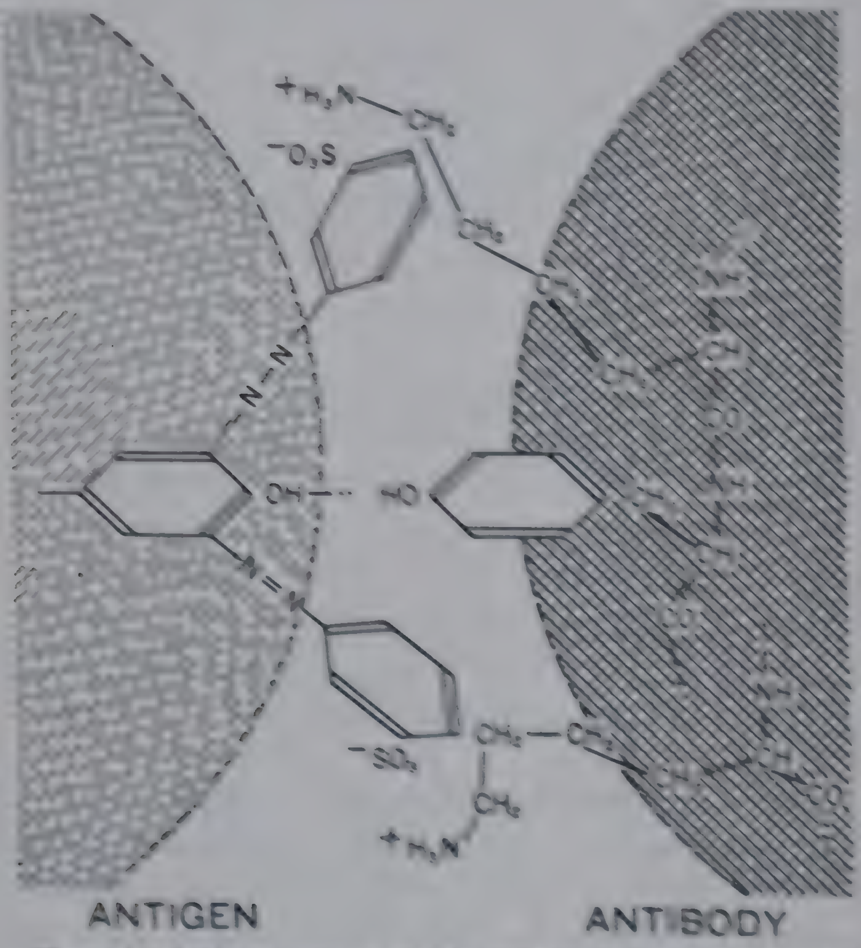


Fig. 24. Hypothetical manner in which surface structure of antibody molecule might correspond to part of the surface of a synthetic conjugated antigen, in this case a native protein coupled with diazotized metanilic acid (71).

would make it possible to differentiate steric isomers. Such an active patch would have to contain at least three reactive chemical groups (98); it would probably contain a good many more.

Marrack (129) has given an interesting picture of how the spatial relationships might account for observed serological differences of optical isomers. The case of antibodies for the α- and β-glucosides (see page 110) is illustrated by Figure 25.

5. The Reversibility of Serological Reactions

It has been found possible to dissociate considerable antibody from certain antibody–antigen complexes (35, 59, 76, 82, 109). The actual proportion recovered depends on the method and the kind of antibody, antipneumococcal antibodies appearing to be particularly easy to recover. Some normal antibodies, such as normal hemagglutinins, human isoagglutinins, and particularly "cold agglutinins," can be separated rather easily from the antigen.

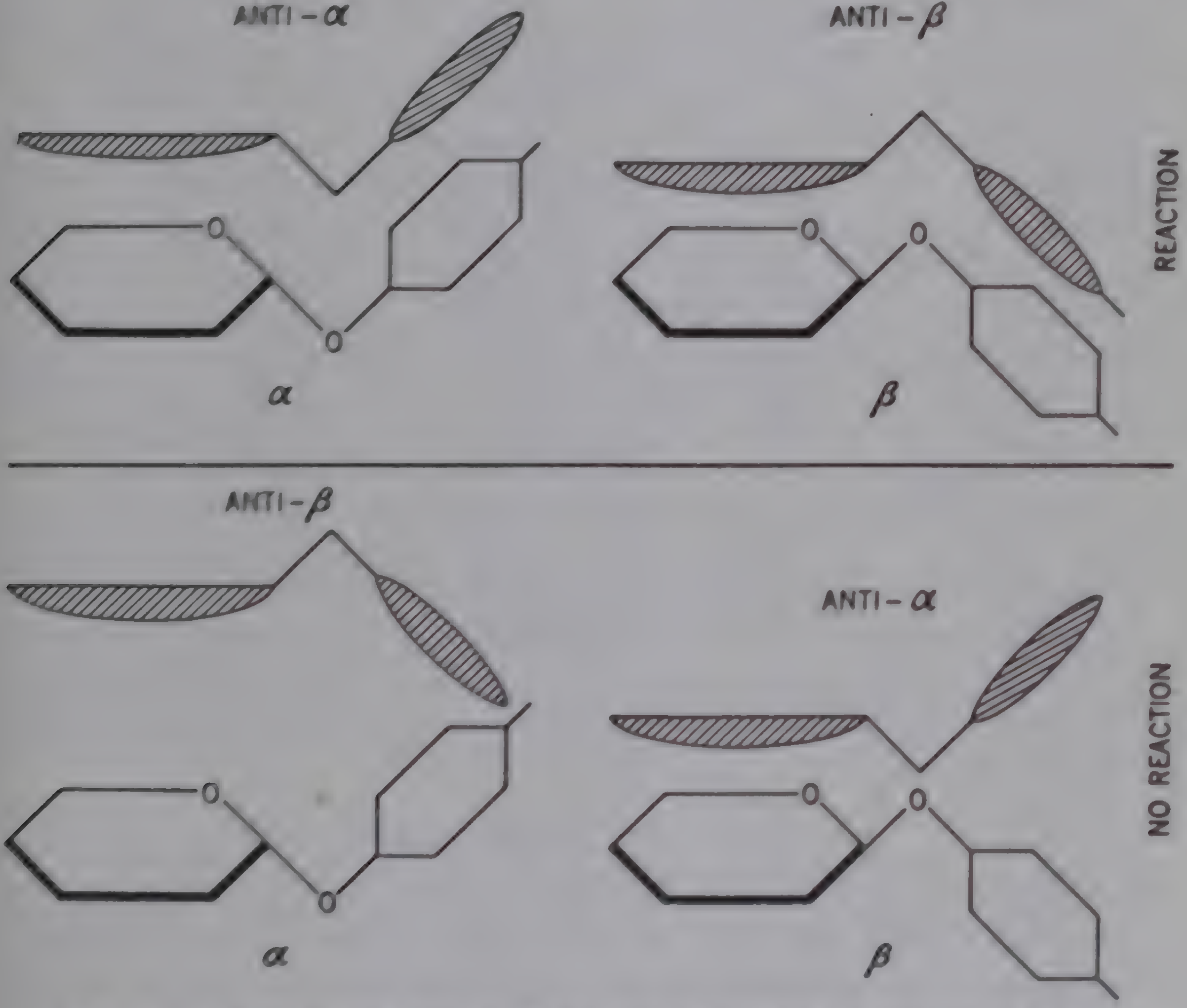


Fig. 25. Hypothetical way in which part of antibody structure might correspond sterically to optically active haptens, in this case α- and β-glucosides (modified from Marrack, 128).

It is a common observation that a mixture of a precipitin and a protein antigen will not result in the formation of a specific precipitate if the antigen is present in too great proportion ("antigen excess"). As would be expected from this, it is found that if excess antigen is added to a specific precipitate, it will dissolve. It is very difficult to see how this could take place except by reversal of some or all of the reactions involved in the formation of the precipitate.

There are some observations in the literature (see 13, 16) suggesting an increasing firmness of union, or "stabilization," between antibody and antigen with the passage of time. Marrack (128) has assumed that antibody and antigen form irreversible compounds. In some cases the evidence for this seems fairly convincing, in others it is possible to think of other explanations for the results obtained. Well-founded or not, the conclusions based on such experiments have probably influenced opinion in regard to the similar but independent question of the solubility of precipitates in excess antigen, which probably involves the reversal of some of the reactions which took place during the formation of the precipitate. Boyd (16) found no evidence that the solubility of ovalbumin–antiovalbumin precipitates was diminished by storage for ten months in the ice box; the reversibility of the reaction, as judged by the solubility (and rate of solution) of the precipitate in antigen, remained unchanged.

Teorell (173), in his quantitative theory of the precipitin reaction, assumes that the reactions are reversible and that equilibria exist between them. The linear relationships obtained when the ratio of antibody to antigen is plotted logarithmically against the concentration of whichever reagent remains in the supernatant (Figs. 26a and 26b) has been offered as an argument for the reversible character of the reaction (19).

6. Rate of Combination

If agglutinin is added to red cells and the tubes are immediately centrifuged, the cells will be found to be practically as strongly agglutinated as if the mixture had been allowed to stand for an hour before centrifuging. However, in some cases the degree of agglutination will be a little less. This suggests that the greater part of the antibody combines in a few seconds, but that some combination may still be taking place after some minutes have elapsed. If we suppose that the union takes place only when a molecule of antibody collides with (or approaches sufficiently near) a molecule or a cell of antigen, this result is not surprising, for it may be calculated that antibody molecules have velocities of the order of half a meter per second at room temperature, due to Brownian motion (26). Cromwell (40) found that the amount of hemolysin fixed by red blood corpuscles did not increase after 15 minutes. Dreyer and Douglas (45), however, found that there was some increase in the amount of agglutinin fixed by bacteria up to four hours. Follensby and Hooker (60) found that

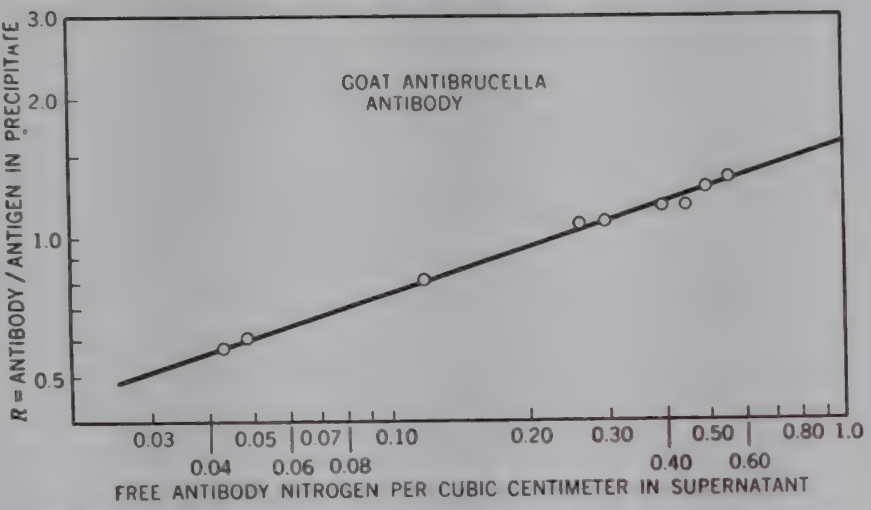


Fig. 26a. Linear relation between ratio of antibody to antigen (R) in precipitate and concentration of free antibody in supernatant, when plotted logarithmically (antibody in excess).

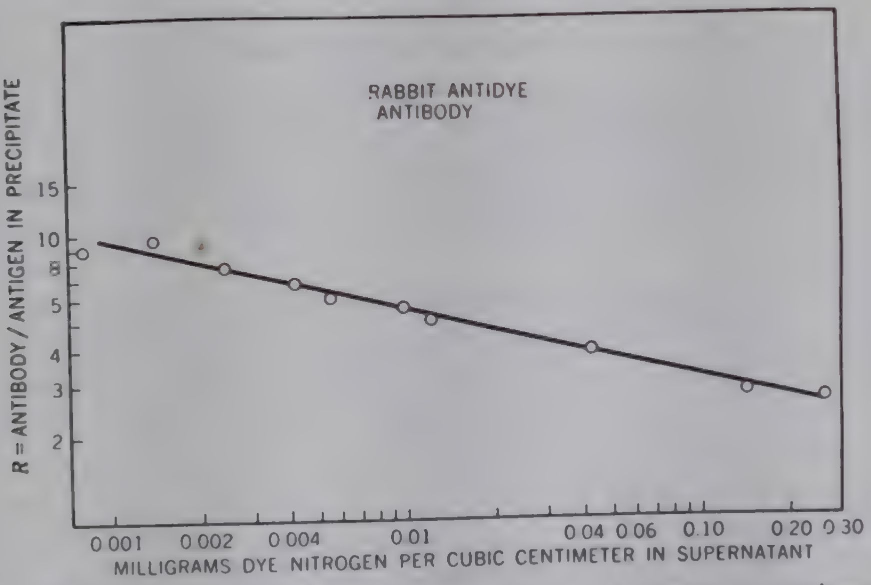


Fig. 26b. Linear relation between ratio of antibody to antigen in precipitate and concentration of antigen in supernatant, plotted logarithmically (antigen excess).

in toxin–antitoxin mixtures 99% or more of the toxin combined in less than ten minutes, probably in only a few seconds. When precipitin and antigen are mixed, the precipitate often appears instantaneously so far as can be observed. Heidelberger, Treffers, and Mayer (87) found that combination between antibody and egg albumin, even at 0°C. was apparently complete in 20 seconds. Eagle (53) states that in the flocculation test for syphilis the combination between the reagin and the lipoid "antigen" is more than 95% complete within one minute after mixing (see 181). Mayer and Heidelberger (132) obtained evidence that combination between pneumococcus polysaccharides and their antibodies is at least 90% complete in less than three seconds at 0°C. The rate at which heat is evolved when antibody and antigen react (see below) also suggests a very rapid reaction.

From these observations it may be concluded that the primary stage of antibody–antigen combination probably goes at first with great rapidity, the velocity falling, perhaps exponentially, with time. The reaction is probably essentially complete, in the majority of cases, in one or two minutes at the most.

7. HEAT OF SEROLOGICAL REACTIONS

If the forces causing antibody and antigen to unite are essentially chemical, and especially if they involve polar linkages, the heat of combination of antibody and antigen should be of a magnitude which appears reasonable in the light of our knowledge of classical thermodynamics.

Using the calorimeter described by Conn, Kistiakowsky, and Roberts, Boyd *et al.* (23) measured the heat evolved at 31°C. when an antibody (antihemocyanin from the horse) reacted with its antigen (hemocyanin of *Busycon canaliculatum*). In the region of antibody *excess*, where no precipitate was formed, a value of about 3.0 calories per gram of antigen nitrogen was found. Since the molecular weight of the antigen was found by Svedberg to be 6,800,000, this corresponds to about 3,300,000 small calories per mole of antigen. It was believed that this value is probably accurate to about 20%. By extrapolation from the results of analyses of specific precipitates, it was calculated that the result corresponds to about 40,000 small calories per mole of antibody. The rate of heat evolution indicated that the reaction was complete within a few (about three) minutes after mixing.

The magnitude of the result would presumably differ with different systems, and if the reagents were mixed in different proportions.

The heat of reaction per mole of antigen is large, as one would expect, since it is the resultant of the heats of reaction of a number of molecules of antibody with each antigen molecule. The heat of reaction per mole of antibody is more interesting, since presumably this results from the reaction of a small number of chemical groups. Boyd *et al.* concluded on the basis of thermodynamic reasoning that the values reported by them were of a reasonable order of magnitude.

8. Effect of Varied Conditions on the First Stage of Antibody–Antigen Reactions

Since the first stage of serological reactions is usually followed by a second stage, it is generally difficult to distinguish between the effects of altered conditions on the two stages. If we make a change in the conditions of a precipitin reaction, and obtain less specific precipitate, for instance, does this mean that less antibody and antigen have combined, or that less of the antibody–antigen compound has precipitated? In only a few cases has it been possible to study the effect on the two stages separately.

Effect of *p*H. Coulter (39) found that the maximal binding of hemolysin took place at *p*H 5.3, near the isoelectric point of globulin. Euler and Brunius (58), working with hemolysin and cell stromata, found similar results. In Coulter's experiments, the reaction was found to be fully reversible, i.e., the same result was reached by putting the cells in hemolysin solutions at various *p*H values, or treating with lysin at one *p*H and then altering the *p*H. DeKruif and Northrop (117) found that agglutinin for typhoid bacilli was taken up best at *p*H values above 6, at *p*H values lower than this less was taken up, and below *p*H 4 the amount was much reduced. Marrack (129) has suggested that this might mean that the combination depends on the degree of ionization of the carboxyl groups.

Effect of Salt. Bordet (13) showed that the presence of salts was necessary for agglutination of bacteria, but that combination occurred without it. Landsteiner and Welecki (123) found that the combination of hemolysin with red cells was considerably reduced by hypertonic (1 *N*) sodium chloride solution. Heidelberger and Kabat (76), following earlier work of Heidelberger and Kendall, found that considerable antibody could be dissociated from specific precipitates and from agglutinated pneumococci by the action of 15% sodium chloride. Duncan (49) did not find much effect of variations in salt concentration on the combination of O agglutinins with smooth non-flagellated strains of intestinal bacteria. A considerable effect was observed with flagellar agglutinin. Combination was stated to take place in the entire absence of salt.

These experiments indicate that combination of antibody and antigen can take place without the presence of salts, and is aided to a certain degree by increasing salt concentrations up to moderate values, beyond which further increase in salt concentrations tends to interfere. Marrack (129) has suggested that the reduced combination of antibody and antigen in high salt concentrations may be due to the establishment of an atmosphere of electrolyte ions around the oppositely charged polar ions of the combining groups of antibody and antigen, reducing the attraction of these for one another.

Effect of Temperature. Cromwell (40) studied the effect of temperature on the combination of hemolysin with red cells. Different sera showed different optimal temperatures (giving maximal combination), ranging from 15°C. to 40°C. It is of course difficult to study serological reactions over any very wide range, because temperatures higher than 60°C. are likely to damage one or both of the reagents.

B. Second Stage: Neutralization, Complement Fixation, Precipitation, etc.

When antibody reacts with most simple haptens, no second stage is observable, and it is likely that the primary stage of combination completes the reaction. When toxins react with antitoxins, the toxin is found to be wholly or partially neutralized, which is probably an immediate consequence of being combined with antibody. But when conditions are right the combination with antibody results, in the case of some haptens and toxins and other antigens, in another, visible phenomenon, the formation of a precipitate. This usually follows the combination of antibody with carbohydrate and protein antigens. Antigenic cells after combination with antibody often stick together in visible clumps, or are observed to be lysed by the action of complement. In spite of rather extensive study, the exact mechanism of these secondary reactions is still a matter of controversy. It will therefore sometimes be necessary in the following exposition to present more than one point of view.

1. COMPLEMENT FIXATION

A discussion of the mechanism of this second stage serological reaction will be more profitably postponed to Chapter VII.

2. LYSIS

The first observations of specific lysis were made with bacteria, but the greater ease of demonstrating the lysis of red blood corpuscles has led to more intensive study of the latter, and it will be advantageous to discuss hemolysis first.

Hemolysis is the release of the hemoglobin from the inside of the erythrocyte. This is called "laking" of the cells. The cell stromata remain undissolved, although altered in size and shape and osmotic properties (14). Nonspecific organic and inorganic substances, or even too low a salt concentration, as well as antibody plus complement, can produce lysis.

The amount of antibody required to sensitize a cell for lysis is extremely small. Brunius (30) calculated that only about 30 molecules of antibody were required to sensitize an erythrocyte for hemolysis, and that the fraction of the surface covered was only about 0.001%. Abramson (1) and Heidelberger *et al.* (88) have also reported observations indicating that very little of the cell surface need be affected to give complete hemolysis. The indicated picture seems to be the production of one or a few "holes" in the cell envelope, thus releasing the hemoglobin.

Specific hemolysis is impossible without the preliminary union of antibody with the cell, but antibody alone does not produce it. The co-operation of other constituents of serum, called collectively complement (or alexin), is needed. The nature of these substances will be considered in the following chapter.

Complement is not specific, for one source of complement may be satisfactory for lysis of a number of serologically different cells by the appropriate antibodies. Guinea pig serum is the commonest source, but complement is present to some degree in other fresh sera. The sera of different species may, however, differ considerably in their complement activity for any particular lytic system.

Complement does not combine with the cell until the latter has been sensitized by combination with antibody. The degree of lysis depends both on the amount of complement available, and on the amount of antibody.

3. BACTERIOLYSIS

Although in some instances, as in Pfeiffer's original observations with the cholera vibrio, gross degenerative changes analogous to hemolysis have been observed in bacteria, observation by the naked eye is not in general satisfactory to detect bacteriolysis. When we speak of measuring the bacteriolytic power of a serum it is the bactericidal effect which we actually measure.

This is estimated by mixing a light suspension of bacteria with complement and antiserum which has been inactivated (heated to 56°C.) to destroy any complement present in it. The proportion of organisms killed is estimated by culturing the surviving bacteria.

Not all bacteria are killed by such a combination of antiserum and complement. The cholera vibrio, the typhoid bacillus, and most Gram-negative bacilli are readily killed and lysed, but other bacteria, such as the Gram-positive cocci, are not susceptible. This is known not to be due to any failure of complement to combine with the sensitized cells.

A prezone is often observed in such experiments, i.e., strong concentrations of serum may fail to kill, while lower concentrations may kill. It may be that when too much antibody is combined with the cell surface, the surface properties are too much like those of uncombined antibody, and complement is not attracted. It has been reported that complement was not fixed when antigen was precipitated with excess of antibody (see Chapter VII). In some such cases, perhaps, although complement combines, the surface is effectively screened from its lytic action by the heavy coating of antibody.

4. NEUTRALIZATION OF TOXINS AND VIRUSES

In the processing of neutralizing toxins, antibody combines with them, in varying proportions, dependent on the ratio in which the reagents are mixed, as claimed by Danysz, denied by Ehrlich, and experimentally shown by Healey and Pinfield (73) and Pappenheimer and Robinson (147) (see Table XXXVI). Ehrlich early demonstrated that an antibody to the vegetable toxin, ricin, would neutralize *in vitro* its hemolytic power. Similarly it has been found that neutralizing antibodies for vaccinia virus can be adsorbed by the elementary bodies (163), which points to union between the virus and the antibody.

It is not known how the combination with antibody neutralizes toxins and viruses. Toxins are evidently not permanently changed by combination with antitoxin, for it has been found that some toxins, snake venoms for instance, can be recovered from the combination by destroying the antitoxin (33, 135, 178). Similarly, it has been shown by Todd (176), Andrewes (6), and others that neutral mixtures of antibody and virus can in some cases be made active again by simple dilution or centrifugation. Perhaps the action of the antibody is simply to cover the toxin or virus and thus prevent it from coming in contact with the susceptible tissue. Salaman (163) suggests that antivirus antibodies act by preventing the entry of the virus into the cell.

Combination of antienzyme antibodies with enzymes often does not destroy or even lessen very much the activity of the enzyme (4). In other cases the activity is inhibited (167). See also (120).

Since the flocculation of toxins by antitoxins was not at first known (being missed because of the narrow zone in which precipitation is obtained), and because the point of primary clinical interest is the toxicity of the mixtures, most of the studies on toxin–antitoxin reactions have been made by mixing toxin and antitoxin in varying proportions and testing the toxicity on animals. It is probable that in such reactions we have an opportunity to study the characteristics of the primary stage of serological reactions, practically independently of the complications introduced by any second stage.

From the nature of the tests made, it was natural to study the relation of the amount of bound toxin to the amount remaining free. The resulting data when plotted give smooth curves. For example, see Figure 27 and Table XXXVI. The calculation of such curves mathematically is discussed in the Appendix to this chapter.

The results of many experiments (11, 40, 57, 65, 66, 126) can be fitted by equations of the adsorption type. One of the most used has been the "Freundlich" adsorption isotherm:

$$R = kx^n$$

in which R is the amount of substance adsorbed per unit of adsorbant, x is the final concentration of the adsorbed substance in the supernatant, and k and n are constants. Such equations apply fairly well to quantitative results obtained by titrating toxins and antitoxins (see Appendix).

Even knowing only approximately the numerical laws governing toxin–antitoxin reactions, we can now see why the early workers were often puzzled by the quantitative relations they observed. From Figure 27, we see that the addition of 0.2 cc. of antilysin to the amount of tetanolysin used lowers the toxicity to about 35% of its initial value. The addition of another 0.2 cc. of antilysin has a much smaller relative effect, for the toxicity is still nearly 15% of the initial value. In the experiment of Table XXXVI (see Appendix), we find that the addition of 0.1 cc. of antiserum has neutralized 30% of the toxin. It might have been expected that the addition of three times as much (0.3 cc.) would neutralize 90%, but actually it neutralizes only 78%, and the addition of 1.0 cc., which we might have expected to neutralize three times as much toxin as was used altogether actually neutralizes only 96% of that present, leaving 4% still free.

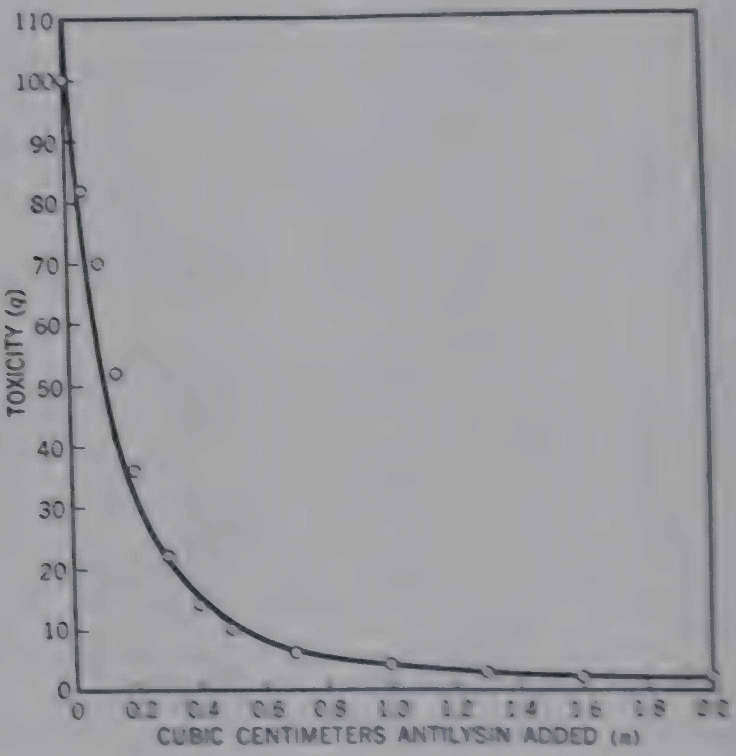


Fig. 27. Relative toxicity (q) of a constant amount of tetanolysin after addition of varying amounts (n) of antibody ($\bar{\gamma}$).

Similarly, Ehrlich found that the amount of toxin which, mixed with one standard antitoxin unit, would just kill a guinea pig, was not twice, but actually of the order of 50 times as much as that contained in the L_0 dose (the amount just neutralizing one unit of antitoxin). Since he did not consider combination in multiple proportions, Ehrlich was led to the unnecessary assumption that toxin contains a variety of substances of different degrees of combining power (or avidity) for the antitoxin.

The bearing of the above facts on standardization of toxins and antitoxins is discussed in Chapter XI.

Danysz Phenomenon

Danysz (41) observed that when toxin is added to antitoxin, the toxicity of the mixture depends partly on the way in which the toxin is added. If an equivalent amount of toxin is added all at once, the mixture is nontoxic, but, if it is added at intervals, in fractions, the final mixture is generally toxic. This suggests that the antibody in the serum was all expended in neutralizing the first portions added, and not enough was left to neutralize the final portion. From the above discussion of toxin–antitoxin combination (and Table XXXVI) it is clear that the explanation of this is, as Danysz thought, connected with the ability of toxin to combine with antitoxin in multiple proportions. If the reaction were reversible, however, we should expect such a toxic mixture, on standing, to become nontoxic; this has been observed (73), indicating the reaction is at least partly reversible. Heidelberger and Kendall (79) reported an analogous experiment, using S3 and antipneumococcus antibody. It has been objected (129, 147) that combination in multiple proportions can hardly be the whole explanation of the original Danysz effect, as in toxin–antitoxin mixtures generally no precipitate forms. Therefore, for the toxin–antitoxin compounds a further change, after combination, to less soluble forms was postulated. This may be correct in some instances, although it might be that low dissociability of the compounds first formed could account for the Danysz effect, without any additional assumptions. This explanation apparently does not satisfy Healey and Pinfield (73) who suggest that any persistent Danysz effect must be due to the toxoid content of the toxin used and the use of *in vivo* methods for the determination of the antitoxin equivalent of the toxin–toxoid mixture.

Avidity

It was fairly soon observed that the *curative* effect of antitoxic sera, as judged by experiments on animals, did not always parallel the antitoxin content as determined by the Ehrlich technic. Kraus suggested that, in addition to the actual content of antitoxin units, antitoxic sera possessed another characteristic which determined the rate of neutralization, and for this he proposed the name *avidity*. It has since been found that the antitoxin content of a serum, as determined by the Ehrlich *in vivo* and the Ramon *in vitro* titrations, are not always the same. An antitoxin may show in the animal several times the protective power which would be expected from its antibody content, estimated by the Ramon test tube titration (see Chapter XI). Glenny (69) and his colleagues have found *in vitro*/*in vivo* ratios varying from 0.4 to 2.0, and have observed that, when the *in vivo* strength seems to be greater, the antitoxin flocculates more rapidly.

Glenny and his colleagues (67) consider however that the important difference is a difference not so much in rate of combination, as in firmness of union between toxin and antitoxin. It is not known if these are related, but it may be suspected that there is some connection. It is apparent that this characteristic of avidity has considerable bearing on the therapeutic value of antitoxins. It is probably for this reason that the Ramon titration, though easier, has not displaced the Ehrlich method for final standardization of antitoxic sera.

With precipitating sera it is often observed that some antisera give rapid flocculation, while others apparently possessing the same content of antibody in terms of milligrams per cubic centimeter flocculate more slowly. Partial absorption of the antibodies also reveals that the antibodies present are not all alike in their flocculative characteristics. It is natural to suppose that these antibodies differ in much the same way as do the antitoxins of different avidity found in antitoxic sera, and we may distinguish different degrees of avidity among them.

In a number of cases (81, 87, 145) serum has been found to contain, in addition to precipitating antibody, antibody which has the power of combining with antigen, but which does not form a precipitate. Such antibody has been variously referred to as low grade, incomplete, and "univalent" (see Chapter II). Also agglutinating sera of nearly the same antibody content may vary widely in the speed with which they agglutinate (20). We might perhaps speak of such differences in antibody as principally differences in avidity. It has been suggested that the differences between these antibodies are due to a smaller number of combining groups on the "low-grade" antibody, but it is perhaps equally probable that they are mainly due to other factors, such as different solubilities, or to less complete specific correspondence between its combining groups and those of the antigen (17, 19a, 19b).

Differences in avidity of hemagglutinating sera have been estimated on the basis of the speed of agglutination (20).

5. Agglutination

If a specific antiserum is added to a uniform suspension of bacteria or red blood corpuscles, the cells are observed, after a certain time, to have stuck together in clumps. In strong reactions these may include practically every cell in the preparation. This is the phenomenon of agglutination. See page 34.

There is no doubt that the primary cause of agglutination is the combination of antibody with the cells; the action of electrolytes seems also to be essential. There are two main theories of the mechanism, that of Bordet (13), who supposed that antibody, by its presence on the surface of the cell, sensitized it to the agglutinating action of the electrolytes, and the "alternation" hypothesis, (74, 104, 129, 150) which supposes that the aggregates are formed merely by the combination of specific groupings on the cell with specific groupings on the antibody (supposed multivalent), which then combines, through other specific groupings, with another cell, and so on. This latter theory assigns no role to any attractive force between the cells, whether sensitized or unsensitized, but supposes that the only force holding agglutinated clumps together is the specific attraction between combining groups of antibody and combining groups of antigen. The relative merits of these two opposing hypotheses will be discussed in the Appendix to this chapter.

Amount of Antibody Involved in Agglutination Reactions

It has been customary to estimate the strength of agglutinating sera by the method of limiting dilution, diluting the serum and keeping the amount of cell suspension constant. This method gives only comparative values and is subject to the uncertainties discussed on page 69. In some cases, as with erythrocytes, it is still the only method available, and consequently has to be used. If due allowance is made for its relative inaccuracy, it can be very helpful. Miles (133) found that if both serum and suspension were diluted, optimal proportion titrations could be carried out as with precipitins and their antigens (see pages 436 and 437). Some success was obtained in differentiating strains of *Brucella* by this method.

The accurate quantitative determination of the actual amount of antibody involved in bacterial agglutination was first made possible by the method of Heidelberger and Kabat (75). They found that pneumococci, in amounts which removed most of the agglutinin from the serum, took up from 0.2 to 1.9 g. of antibody nitrogen per gram bacterial nitrogen. Smaller amounts, perhaps of the order of 1/100 of these amounts, will still bring about agglutination.

Jones and Little (111) found that the volume of agglutinated bacteria was increased by amounts which ranged from values too small to measure to 60–80% of the original volume. It will be seen that these results are in fair agreement with those found by Heidelberger and Kabat, but Jones and Little in their own determinations did not find enough protein taken up by the organisms to account for the observed volume increases, but found instead only one-fourth to one-sixth as much as expected. They suggest that possibly the excessive volume increase is due to nonuniform distribution of antibody over the surface of the bacteria, preventing close packing of the bacteria when centrifuged.

Zones in Agglutination

If an agglutinating serum is sufficiently diluted, it no longer agglutinates the cell suspension. If we view the array of test tubes in which the tests are carried out, with the serum dilutions increasing towards the right, then we shall find that at some point on the right we enter a "zone" in which agglutination is absent, due to the absence in those and succeeding tubes of sufficient antibody to cause the cells to agglutinate (Fig. 28).

With some antisera a more puzzling observation is made. In addition to the zone just described, which we may call the "postzone," we find that at the left, just where the serum concentrations are highest, agglutination fails. This is called the prezone, or "prozone." Coca and Kelley (36) found one such serum (against *Klebsiella capsulata*) which showed this phenomenon to a very marked degree. This serum would even suppress the agglutinating action of other antisera for *Klebsiella*, when it was added to them. The inhibiting action was specific. The inhibiting substance could be absorbed out by treating the serum with homologous organisms, leaving considerable agglutinin; on storage the inhibitory property diminished and finally disappeared. It has also been found possible to give such properties to antisera by artificial means, as by heating. Jones and Orcutt (112) made a careful study of the prezone in bacterial agglutination. They found that it was apparently due to a film of some substance, presumably a globulin, which interfered with agglutination by reducing the cohesiveness, for direct measurement of the cohesive force between smears of bacteria showed that the presence of this film reduced the cohesion to the value for normal organisms, whereas the surface charge did not seem to be altered significantly. Such treated films, after washing, regained the cohesiveness of others treated with non-inhibitory serum, and treated bacteria, after washing, would then agglutinate. It is interesting that no extra increase in volume was observed to accompany the presence on the sensitized cell of this inhibiting film, so it would seem that the film is either very thin, or consists of a few molecules in scattered loci on the bacterial surface. Treatment of agglutinating sera in ways which destroy their agglutinating activity does not always produce such inhibiting antibodies (19a, 19b).

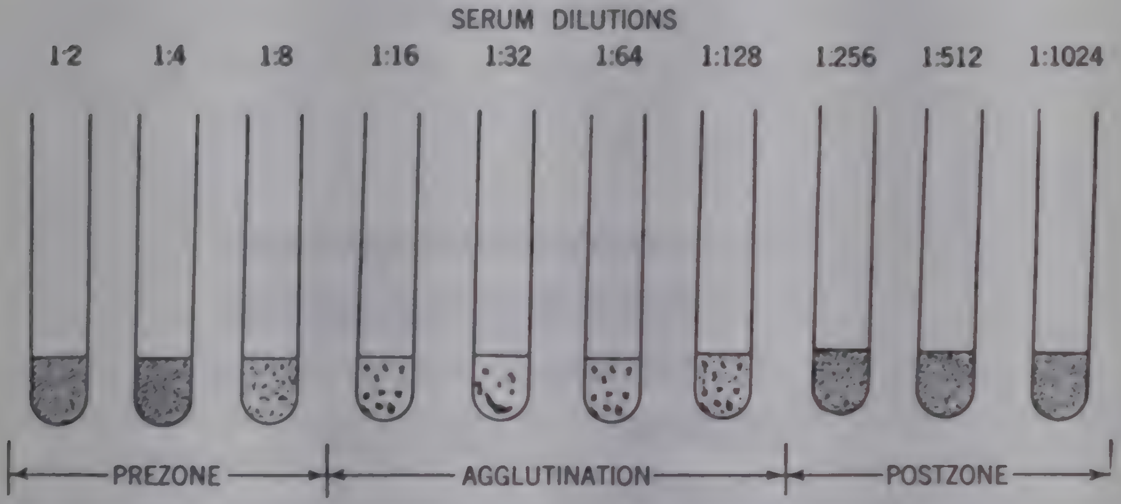


Fig. 28. Diagrammatic representation of zones in agglutination.

Effect on Agglutination of Changes in Conditions

Salts. Since salts have a flocculating action on suspensions, it could be suspected that salt will be necessary for agglutination, and this is found to be so. Higher concentrations diminish agglutination again, so that an "optimum" is obtained for salt concentration, as well as for serum concentration.

Duncan found that all three variables, serum concentration, density of suspension, and salt concentration, were operative in determining optimal agglutination. With the latter two constant, there is a certain antibody concentration that gives maximal sensitization of the bacteria and the greatest degree of cohesiveness. With the first and third constant, there is a certain density of suspension which provides enough nuclei of aggregation, and yet allows all the cells to be adequately sensitized. With the first two constant, a salt concentration is found where the amount of antibody combined with each cell is a maximum, and greater concentrations of salt diminish the cohesiveness of the sensitized cells (see 77).

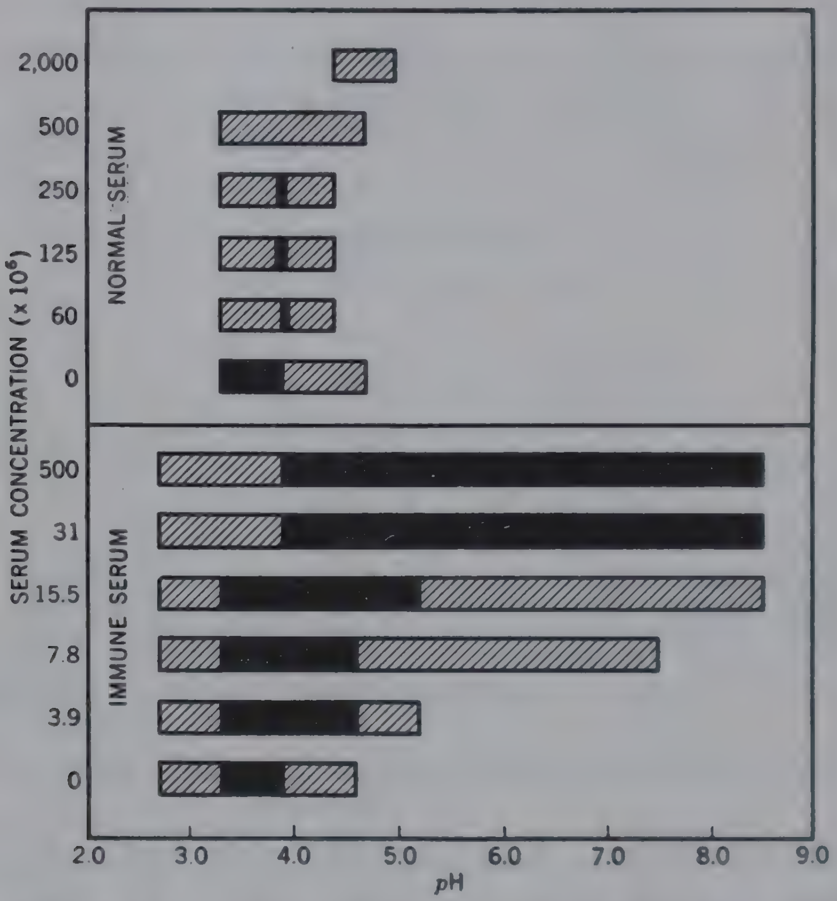


Fig. 29. Effect of *p*H on agglutination of typhoid bacilli by normal and by immune sera (144). Black areas indicate complete agglutination; shaded areas, partial agglutination.

Effect of *p*H. With higher antibody concentrations complete agglutination occurs over a wide range of *p*H; with lower concentrations the effect merges into that found with normal serum and other proteins, where nonspecific agglutination is found at low *p*H levels. The measurements of Northrop and de Kruif (144) are shown graphically in Figure 29.

The shift of the optimal *p*H towards lower levels, when the antibody concentration is reduced is stated by Marrack (129) to be accounted for by the low isoelectric point of the bacteria. The optimal *p*H of the red cells studied by Coulter (38) was higher than that for bacteria even in the case of unsensitized erythrocytes. For minimally sensitized cells he found a value of about pH 5.3.

Effect of Temperature. The rate of agglutination usually increases rapidly from 0° to 30°C. Above 30° the increase is less rapid, and the speed may even fall off (52). Above 56° the antibody begins to be affected. It is likely that the increased speed with higher temperatures is due to the increased rate of Brownian motion which results in more collisions per unit of time.

Shaking, Stirring. Shaking and stirring have an accelerating effect on agglutination similar to that on precipitation. Further details will be found in the sections devoted to the latter reaction.

Nonspecific Serum Proteins. As Marrack (129) points out, in view of the protecting and precipitating effects which proteins exert on various suspensions, it would be expected that nonspecific proteins may affect agglutination and flocculation, and it would be expected that they might either promote or partially inhibit these reactions. The effect is generally not very great. Dean (43) found agglutination increased by euglobulin from guinea pig serum. Eagle (51) found a fraction of fresh normal serum precipitated by dilution and carbon dioxide accelerated agglutination. He found this to parallel the complement "midpiece" activity (see Chapter VII). Duncan (49) found a retarding action of normal serum. Maltaner and Johnston (127a) have suggested that the effect of fresh serum on the rate of agglutination is due to residual fibrinogen. The increased rate of sedimentation of erythrocytes observed in certain clinical conditions such as infections results from "rouleaux" formation, not true agglutination.

6. The Precipitin Reaction

The formation of a precipitate when antibody and soluble antigen are mixed is one of the most striking phenomena of serological reactions; some of the older and many of the more recent studies of it have yielded results of great interest and importance.

Amount of Precipitate; Zones

Either the amount of antiserum or the amount of antigen can be varied; it is usual to keep the concentration of one reagent constant, and add various amounts of the other. Since the precipitates obtained are now usually analyzed by some modification of the micro-Kjeldahl technic developed by Parnas and Wagner (148), it is advisable to adjust the amounts of both reagents so as to obtain the appropriate amount of nitrogen (or of any other constituent being estimated in the precipitate). Heidelberger and MacPherson (85) have described a method of estimating very small amounts (as little as 10 γ) directly as protein. From the results obtained by either procedure we can calculate back to the basis of constant amounts of serum or of antigen. A series of such determinations made with different proportions of reagents gives a system of data which if plotted in three dimensions will yield a surface which shows how the composition of the precipitate depends on the concentrations of the two reagents; two dimensional graphs can be made which are really just appropriate sections of this.

Most of the precipitating antisera that have been studied quantitatively thus far were obtained from the horse or the rabbit (Pennell and Huddleson used goat serum). In the horse there seems to be a striking difference in the behavior of antibodies against proteins (such as antitoxins) and antibodies against bacterial polysaccharides; in the rabbit this difference is not found, but both kinds of antibodies behave more or less alike, resembling in their precipitating behavior the anticarbohydrate antibody of the horse.

As already mentioned in Chapter II, with horse antisera to proteins it is generally found that, if more than a certain relative amount of antibody is added, no precipitate is produced, soluble compounds being formed, whereas carbohydrates are precipitated even with large excess of antibody. In the language of our discussion of agglutination, horse antiprotein sera exhibit a prezone. Rabbit antibodies, whether against protein or carbohydrate, do not show a prezone with excess antibody. It may be suspected that the difference is at least partly due to a difference in solubility of the various antibodies, for it will be recalled that the anticarbohydrate antibodies in the horse, and most antibodies of the rabbit, are predominantly found in the euglobulin (least soluble) fraction of the serum proteins, while the antiprotein antibody in the horse is mostly of pseudoglobulin (more soluble) character.

All precipitating systems exhibit the phenomenon of the postzone which is unfortunately usually called the prezone (since in precipitin reactions it is usually the antigen which is diluted, so that the strongest antigen solutions come first) (see Fig. 35); for this reason and for

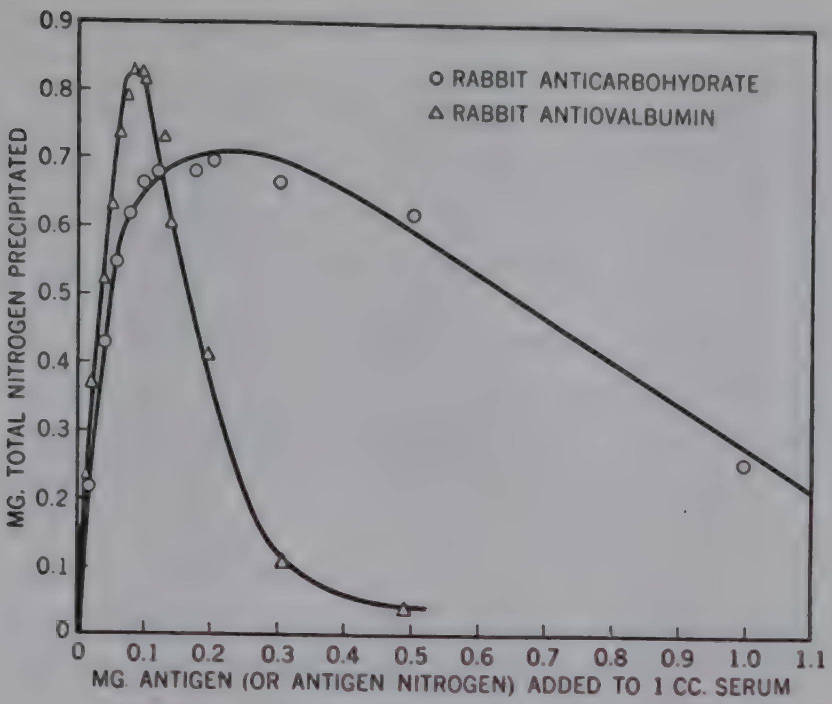


Fig. 30a. Relation between total nitrogen precipitated from one cubic centimeter of rabbit antiserum and amount of antigen added (81, 83).

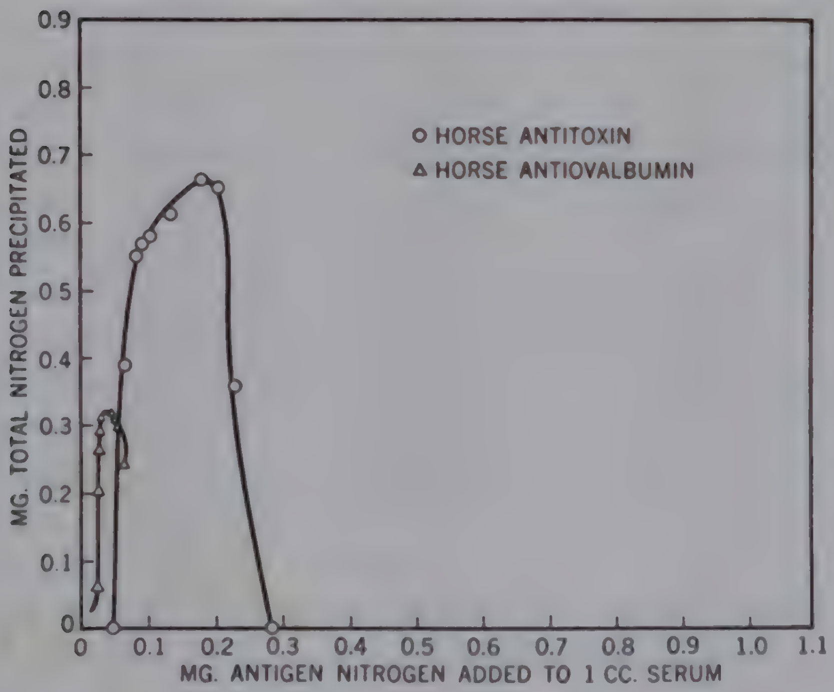


Fig. 30b. Relation between total nitrogen from one cubic centimeter of horse antiprotein serum and amount of antigen added (105, 145, 147).

others, it seems best to use Heidelberger's term "inhibition zone." We may define this as the zone in which there is insufficient antibody to precipitate the antigen present. The antibody in this zone forms soluble compounds with the antigen.

The actual amount of precipitate obtained from a given amount of antiserum depends in the first place on the amount of precipitating antibody present, and in the second place on the amount of antigen added. As increasing amounts of antigen are added, the amount of precipitate increases up to a maximum, then declines as the inhibition zone is reached. This is brought out by Figures 30a and 30b (drawn to the same scale), which are based on results of Heidelberger and Kendall (81, 83), Pappenheimer and Robinson (147), Pappenheimer (145), and Hooker and Boyd (105). Figure 30a illustrates the typical results obtained with rabbit antisera, and Figure 30b those with horse antiprotein sera. As has already been mentioned, horse anticarbohydrate sera have been found to behave in this respect much like rabbit antisera. The precipitating zone may be somewhat broader, however, in some cases, with rather a flat plateau on top (74, 79). We may mention also that horse antiprotein sera, when sufficiently strong, may show a much broader zone of precipitation than those shown in Figure 30b; the breadth of the zone may even exceed (105) that of the rabbit sera shown in Figure 30a.

If the supernatants from such precipitates are tested for antibody and antigen, it is found that antibody is present in the first tube (where insufficient antigen was added), and antigen is present in the last tubes (where excess antigen has been added). In the central part of the range one or more tubes will be found where there is either no antibody and no antigen, or small traces of both, in the supernatant. This is called the equivalence zone (Heidelberger). In some systems this zone may coincide with, or lie near, the point of maximal precipitation of antibody. In other systems (80, 127), however, the point where the maximal precipitate is obtained may lie in the region of antigen excess.

It has been found convenient to divide the whole range of the reaction of rabbit precipitin with antigen into the following five zones in order of increasing antibody to antigen ratio: complete inhibition, partial inhibition, antigen excess, equivalence, and antibody excess. In the case of horse antiprotein precipitin it would be necessary to add two more zones, partial antibody inhibition and complete antibody inhibition. A schematic presentation of this arrangement, which also serves to indicate approximately the meaning to be attributed to each term, is given in Figures 31a and 31b.

Composition of the Precipitate

The precipitate contains both antibody and antigen; with the majority of antigens the greater part of the precipitate consists of antibody; with the antigens of high molecular weight the reverse may be true. Precipitates may contain several per cent of lipoid; in addition minor constituents such as complement are usually present (see Chapter VII). There is definite evidence that the washed precipitate does not contain any appreciable amount of nonspecific serum proteins (79, 105, 130).

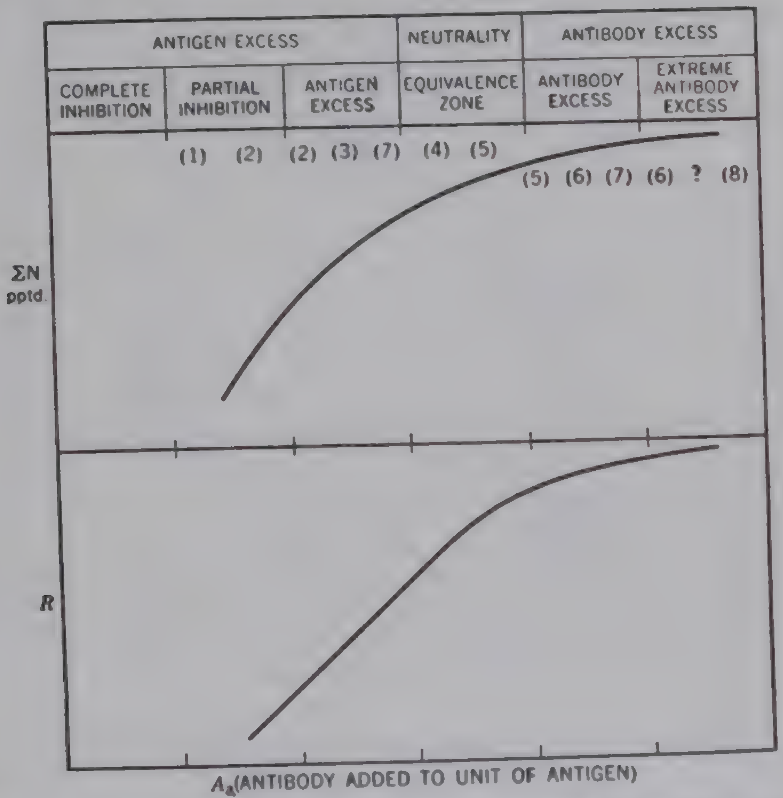


Fig. 31a. Zones into which the precipitin reaction with rabbit antibody can be divided. Curves show schematically the relation between total nitrogen precipitated (above) and ratio of antibody to antigen (R) in the precipitate (below) observed when increasing amounts of antibody (A_a) are added to a constant amount of antigen (G).

The antibody and antigen are found in the precipitate in varying proportions, depending on the ratio in which the serum and antigen solution are mixed (and also on the final concentration in the supernatant of the reagents, see page 268) and on other factors such as the molecular weight of the antigen.

Influence of Molecular Weight of the Antigen

Zinsser (186) showed that the relative surfaces involved would explain the well known observation that less antibody is required to agglutinate bacteria than to precipitate, for example, a protein antigen. Boyd and Hooker (24, 27) called attention to the fact that the ratio of antibody to antigen in precipitates made at the equivalence point (midpoint of equivalence zone) depends on the molecular weight of the antigen. The dependence is not absolute, as will be seen in Figure 33, which shows the data up to 1940, but it was found that there was at least a highly significant degree of correlation between the two variables. Boyd and Hooker interpreted this tentatively as due to the fact that at the equivalence point the surface of the antigen molecule is just covered with molecules of antibody, each such molecule supposedly acting as a chain of three (or better, four) roughly spherical units of molecular weight about 35,000 (Fig. 32).

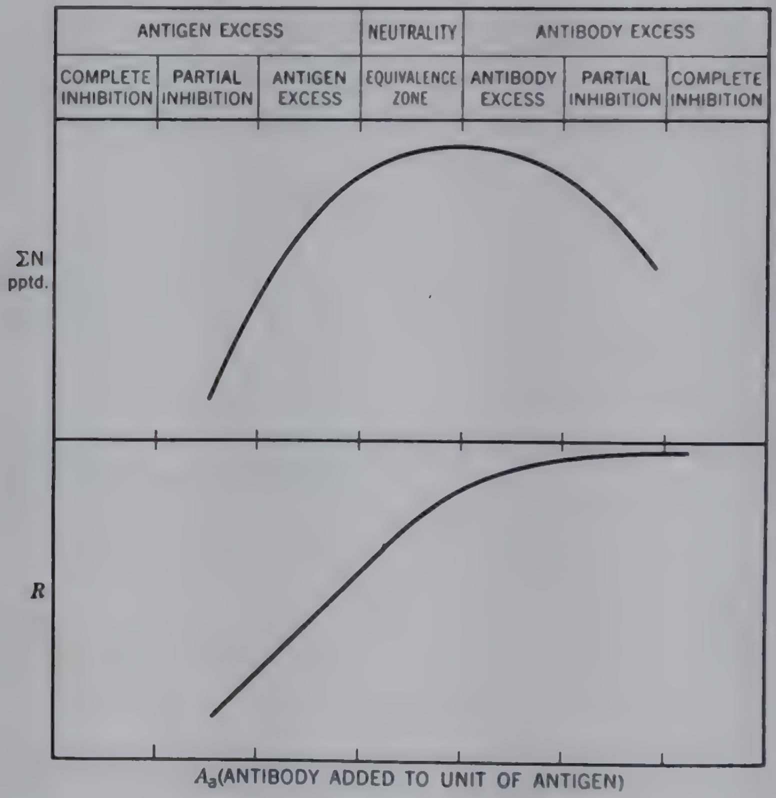


Fig. 31b. Zones of the precipitin reaction with horse antiprotein antibody.

It would do equally well for this purpose, and perhaps accord better with our ideas of the shape of antibody molecules, to suppose that antibody behaved as a long flexible ellipsoid. On the basis of such a picture it was possible to calculate by spherical geometry that the theoretical relation between molecular weight of the antigen (M) and ratio by weight of antibody to antigen (R) in the precipitates ought to be approximately:

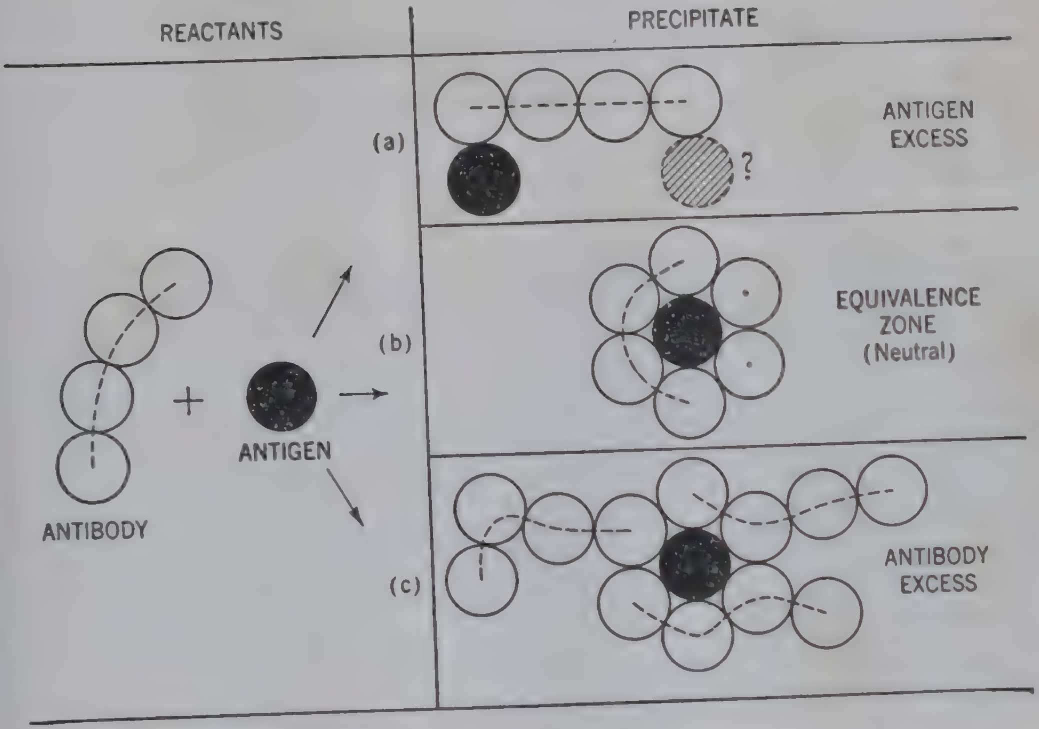


Fig. 32. Hypothetical mode of combination between antibody—assumed to consist of four linked units of about 40,000 molecular weight each,—with an antigen of about the molecular weight of egg albumin (about 40,000), in three different zones of the precipitin reaction. Light shading of second antigen molecule in (a) indicates lack of evidence that antibody can combine simultaneously with two molecules of antigen (25).

$$R = 37{,}800\ M^{-0.8} + 179\ M^{-0.35}$$

In Figure 33 this theoretical relation, shown by the solid line, is seen to fit the data rather well, considering the roughness of many of the measurements. It is doubtful, however, if we are justified in considering the polysaccharide molecules, here plotted at the value, $M = 4{,}000$, to have molecular weights this low, or to be spherical, so it may be that part of the agreement is fortuitous. Also it is perhaps doubtful if antibody molecules are as flexible as required by the above picture. In any case, it can be seen that the ratio does tend to diminish with increasing molecular weight of the antigen, falling from values of around ten for antigens of molecular weight 35,000–40,000, to values of less than 1.00 for the large hemocyanin molecules of molecular weight of about 7,000,000–10,000,000.

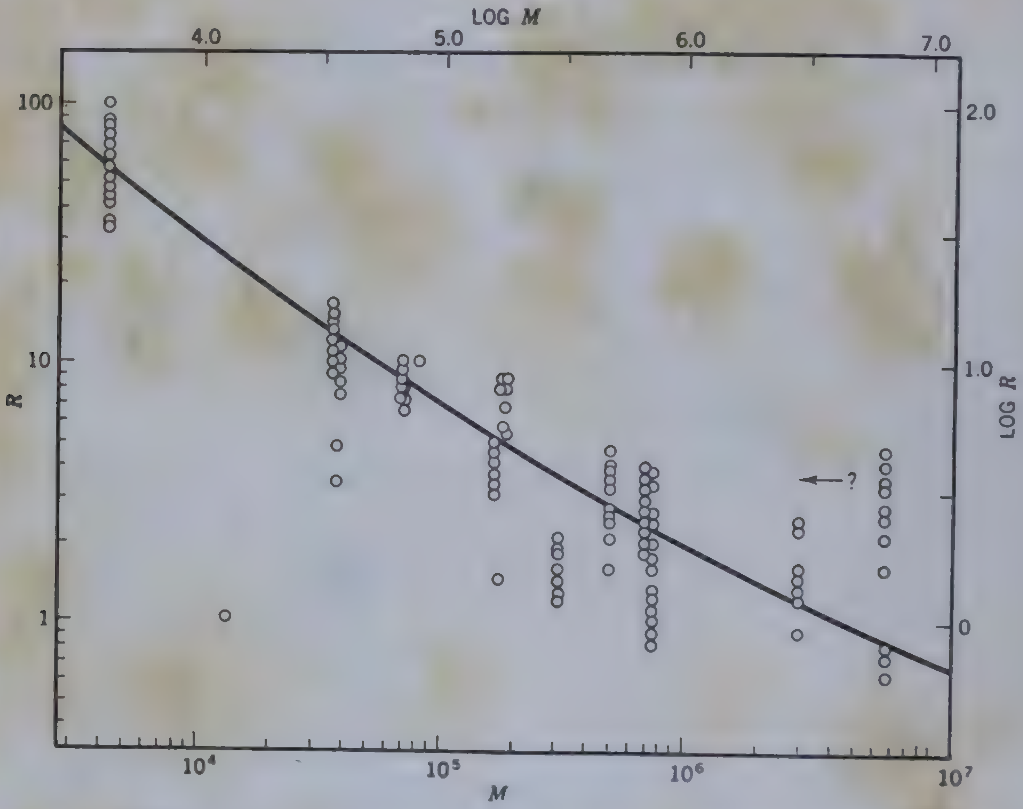


Fig. 33. Calculated relation between ratio of antibody to antigen (R) in precipitates, and molecular weight (M) of antigen, shown by curve (24, 27). Experimental values shown by circles.

Landsteiner (120) has pointed out that this model, however improbable it may be in some ways, predicts a coating of antibody of about the thickness which is actually found (about 40 Å.). See page 243. How (108) derived an improved formula giving somewhat better agreement with the data.

When less than the equivalent amount of antibody is used, the precipitate contains less antibody, and we may suppose the surface to be only partly coated. When more antibody is used, the ratio of antibody to antigen in the precipitate goes up. If antibodies are really to be visualized as just described, it is necessary to suppose that some of the molecules in this case are not in full contact with the antigen surface, and when there is considerable excess of antibody we should have to visualize them as "standing on their heads" (25). This is illustrated in Figure 32. Combination of this type has also been proposed by Heidelberger (74) and by Anderson and Stanley (5). Electron micrographs of tobacco mosaic virus agglutinated by antibody seem to indicate a spacing between the molecules of antigen approximately equal to the length of an antibody molecule. The antibody molecules cannot be seen, being too thin to be resolved by present instruments (Fig. 34).

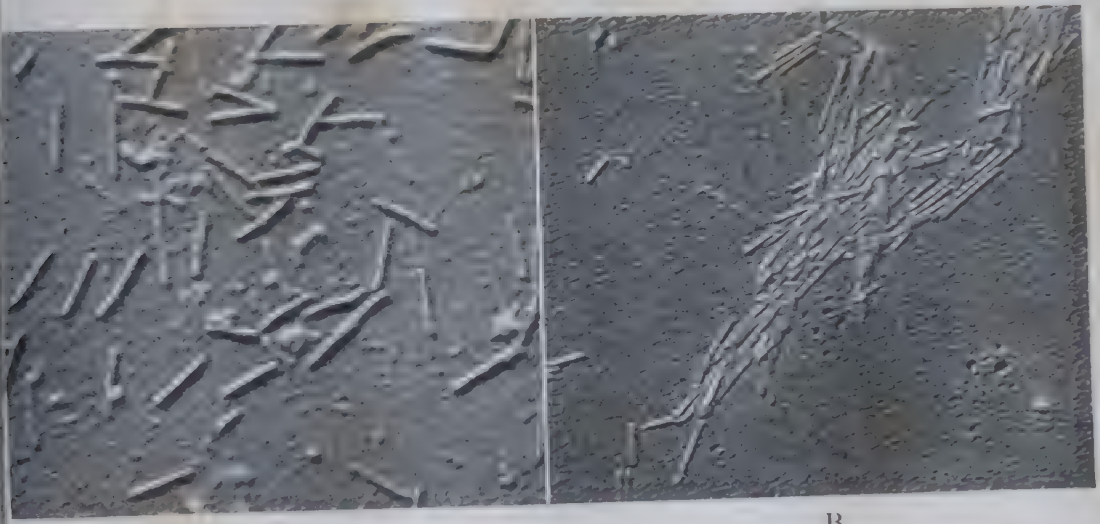

A B

Fig. 34. Electron micrographs of tobacco mosaic virus molecules (A) and tobacco virus molecules precipitated by antibody (B) (courtesy of Dr. W. M. Stanley and Dr. S. Malkiel) (× 25,000).

Influence of Proportion in Which Reagents Are Mixed

It was pointed out (page 235, Fig. 26a, Table XXXVII) that the composition of the precipitate depends, in a given system, on the final concentration of antibody or antigen in the supernatant, as would be expected if an equilibrium is reached. We can draw some conclusions as to the form of this relationship.

We have already stated that the quantitative course of many complicated reactions can be satisfactorily expressed by the "Freundlich" adsorption isotherm:

$$\log R = \log a + b(\log p)$$

where R is the ratio in which the two reagents combine, a and b are constants, and p is the equilibrium concentration of the reagent in excess. Other reactions follow better the quite similar Langmuir adsorption isotherm:

$$\log R = \log ab + \log [p/(1 - ap)]$$

where the symbols have the same significance. It is now known that the finding that a reaction obeys one of these "adsorption" equations does not indicate that the reaction can not be strictly stoichiometric; in fact, Hitchcock (97) has shown that an expression formally identical with the second expression easily can be derived for a simple chemical reaction from the usual assumptions of the mass law. The Langmuir equation was derived by an application of the kinetic theory of reactions (124); Fowler (61) has shown that the equation can be derived, quite apart from any assumptions as to mechanism, from the postulate that each step of the reaction is reversible. Henry (89) has shown that a theoretical derivation of the Freundlich equation is also possible.

It would not be surprising, therefore, to find that the composition of the precipitate and equilibrium concentration of antibody or antigen in the supernatant were related mathematically in a similar way. From Figure 26 it would seem that the relation is in fact of such a type, since a straight line is obtained by plotting the data logarithmically. This suggests, as already mentioned, that the relation may be expressed mathematically by the equation just referred to as the Freundlich isotherm.

Few direct measurements of such equilibrium concentrations of antibody or antigen have been made, however, because of analytical difficulties, whereas we can easily make precipitates by mixing antibody and antigen in various known proportions. Therefore it is desirable to investigate also the way in which the composition of the precipitate depends on the ratio in which the reagents are mixed initially.

As just mentioned, when mixtures are made containing more antibody than required for equivalent proportions, the resulting precipitate will be richer in antibody. The ratio of antibody to antigen in the precipitate does not continue to increase at the same rate, as more and more antibody is used, but the curve flattens out, and the ratio appears to approach an upper limit asymptotically. In the case of most horse antiprotein sera these compounds with higher antibody/antigen ratio are soluble, and precipitation does not occur with any considerable excess of antibody. With other antisera the compounds of higher ratio are all insoluble and therefore precipitate.

If we keep the amount of antibody constant, and add varying amounts of antigen, we find that more than the equivalent amount of antigen gives compounds with an antibody/antigen ratio lower than that formed in the equivalence zone. Below a certain point these compounds are soluble, and no precipitate is formed, in the case of all systems. This gives rise to what we may refer to as the "zone of complete inhibition," or prezone. The ratio of antibody to antigen in the *precipitate* therefore never falls below a certain limiting value, which if it could be measured would give the approximate composition of the compound having the smallest proportion of antibody which will still precipitate. For small antigen molecules, this is estimated at about two molecules of antibody to one of antigen, but for larger molecules as many as 30–40 molecules of antibody may be required to precipitate one molecule of antigen. This is shown in Table XXXIV.

The adjoining Table XXXIII gives two examples of the variation in the composition of precipitate with variation in the proportion in which the reagents were mixed. The first, from Heidelberger and Kendall (79), shows the results for the system horse antipneumococcus type 3 and the specific polysaccharide S3. The second, from Pappenheimer and Robinson (147), is from a study of the reaction of diphtheria toxin and horse antitoxin.

In both of the examples in Table XXXIII, as in the majority of the early quantitative studies on the precipitin reaction, the ratio of antibody to antigen was calculated on the assumption that all the antigen was precipitated, in the absence of any way to determine the amount of antigen directly in the precipitate. This has been shown to be allowable, but it precludes a study of the region where only part of the antigen is precipitated (region of antigen excess). For a study to be carried any distance into the region of antigen excess, it is necessary to have some way of determining the antigen separately from the antibody. When the proportion of antigen remaining unprecipitated is small, serological methods (81, 101) can be used to estimate it, but the only analytical methods of general applicability to this problem depend on the use of antigens which are colored, or contain an inorganic "tracer" atom which can be tested for chemically, or which contain some introduced prosthetic group which enables them to be detected (80, 102, 127, 168, 183, 184). There have been only two investigations which were at all extensive into the region of antigen excess (80, 127).

TABLE XXXIII

RATIO OF ANTIBODY TO ANTIGEN IN PRECIPITATES MADE WITH VARYING PROPORTIONS OF ANTIBODY TO ANTIGEN FOR TWO DIFFERENT SYSTEMS

Horse antipneumococcus S3				Horse antidiphtheria toxin			
S added (mg.)	Σ N pptd. (mg.)	$R = (N/S) \times 6.25$	Reagent present in excess	Toxin N added (mg.)	Σ N pptd. (mg.)	R = antibody N/toxin N	Reagent present in excess
0.02	0.45	140	A	0.023	0		A
0.04	0.79	124	A	0.046	0		A
0.05	0.97	121	A	0.069	0.386	(6.9)	(A)
0.06	1.08	112	A	0.081	0.554	5.8	(A)
0.08	1.29	101	A	0.092	0.564	5.1	
0.10	1.54	96	A	0.103	0.579	4.6	
0.12	1.68	87	A	0.138	0.612	3.4	
0.15	1.71	71	A	0.184	0.661	2.6	(T)
0.20	1.75	?	S	(0.196)		(2.4)	(T)
				0.207	0.652		(T)
				0.230	0.359		T
				0.276	0		T

One cubic centimeter of antiserum used for all experiments. A = antibody, T = toxin, S = specific capsular polysaccharide of type 3 pneumococcus, N = nitrogen. Figures in parentheses somewhat uncertain.

Broadening of the Equivalence Zone

It has been found by Heidelberger and Kendall (81) and Heidelberger, Treffers, and Mayer (87) that the equivalence zone is broader in sera from animals after prolonged immunization than in sera from animals after a brief course of injections. Malkiel and Boyd (127) made an observation which is probably related, that the difference between the midpoint of the equivalence zone and the point of optimal proportions was greater in the sera of animals having had longer courses of injections. All of these workers observed that the ratio of antibody to antigen in precipitates from sera from such hyperimmunized animals might be higher. Heidelberger *et al.* (87) are inclined to attribute this to "multivalency" (hypothetical power of the later antibodies to combine with an increasing number of groupings on the antigen molecule).

Molecular Composition of Precipitates

The application of quantitative methods to the study of the precipitin reaction, plus the determinations of molecular weights of various proteins, including antibodies, by the ultracentrifugal technic, have enabled the calculation of the actual molecular composition of precipitates made under various conditions. In Table XXXIV are given the results of the studies of Heidelberger (74) and collaborators, to which have been added results calculated from the results of Malkiel and Boyd (127), Pappenheimer (145), Heidelberger, Treffers, and Mayer (87), Pappenheimer, Lundgren, and Williams (146) and Kabat (113).

TABLE XXXIV

MOLECULAR COMPOSITION OF SPECIFIC PRECIPITATES

Antigen	Antibody	Empirical composition of precipitate at				Composition of soluble compound in zone of partial inhibition
		Extreme[a] antibody excess	Antibody excess end of equivalence zone	Antigen excess end of equivalence zone	Zone of partial inhibition	
Ovalbumin	Rabbit	A_5G	A_3G	A_5G_2	A_2G	(AG)
Dye-ovalbumin	Rabbit	(A_5G)	(A_3G)	A_5G_3	A_3G_4	(AG_2)
Serum albumin	Rabbit	A_6G	A_4G	A_3G	A_2G	(AG)
Thyroglobulin	Rabbit	$A_{40}G$	$A_{14}G$	$A_{10}G$	A_2G	(AG)
Viviparus hemocyanin	Rabbit	—	$A_{120}G$	$A_{83}G$	$A_{35}G$	—
Tobacco mosaic virus	Rabbit	$A_{900}G$	$A_{450}G$	—	—	—
Diphtheria toxin	Horse	A_8G	A_4G	A_3G_2	AG	(AG_2)
Ovalbumin	Horse	(A_4G)	A_2G		AG	(AG_2)

A = antibody, G = antigen.
Formulas in parentheses are somewhat uncertain.
[a] For meaning of this and similar terms, see Figures 31a and 31b.

From Table XXXIV it may be concluded, since the ratio of antibody to antigen in precipitates is observed experimentally to vary continuously, that, if the above formulas really represent the composition of all the various antibody–antigen compounds possible, the precipitate obtained at any particular point is likely to be a mixture of two or more of these compounds.

Solubility of the Precipitate

It is the custom of all workers to wash precipitates two or three times with saline before analysis. The amount of serum protein removed during these washings may be two or three times the amount remaining in the precipitate, but, by tacit agreement, only that which remains after washing is defined as antibody. Later washings remove comparatively little from the precipitate, in other words, the solubility of the precipitate is very small. Various workers (see references in 129) have found solubilities of the order of 0.05–0.005 mg. nitrogen per cubic centimeter saline; the solubility found with the first washings is larger, falling asymptotically towards a minimum as washing is continued. This suggests that the precipitates are not homogeneous, as we mentioned above. There is perhaps also the possibility of gradual denaturation, but such precipitates still dissolve in excess antigen. Typical results (15) are shown in Table XXXV.

TABLE XXXV

SOLUBILITY IN SALINE (MILLIGRAMS NITROGEN PER CUBIC CENTIMETER) OF THRICE-WASHED PRECIPITATES (HEMOCYANIN OF *Busycon canaliculatum* AND HORSE ANTI-*Busycon*)

Extraction	Volume of extractant (cubic centimeters saline per gram precipate nitrogen)		
	333	*878*	*1660*
1	0.0379	0.0210	0.0211
2	0.0169	0.0032	0.0036
3	0.0082	0.0038	0.0024
4	0.0056	0.0021	0.0026
5	0.0036		0.0011
6	0.0004		0.0001

The question arises: does the precipitate dissolve in these experiments in the form of undissociated compound, or is the dissolved precipitate more or less completely dissociated into antibody and an antibody–antigen compound containing less antibody? This is a difficult question and it has generally been ignored. The available tests for antigen and antibody are often not delicate enough to detect the small amounts of dissociation which might be expected to occur. There surely must be a possibility of dissociation, otherwise the reactions would not be reversible; evidence that they are reversible has already been presented (page 215). A further difficulty arises because of the general nonhomogeneity of antibodies. Even in those experiments in which both antibody and antigen are detected in the supernatant, and in which pure antigens were used (which is not always the case), it is hard to be sure that antibody found in solution is the same as that which precipitated. In fact, according to Heidelberger (personal communication), there is plenty of evidence that the antibody in solution is different. One can only observe that some workers have found both antibody and antigen in the supernatant in the equivalence zone, while others have failed to find either. Different systems may vary in this respect.

Effect of Altered Conditions on Amount and Composition of Precipitate

Electrolytes. Diphtheria antitoxin and toxin do not flocculate in the absence of salt (see page 255) but do combine (60). It has been found (84) that as sodium chloride concentrations were increased from 0.1 M to 1.79 M (physiological saline is about 0.15 M) the amount of nitrogen precipitated from antipneumococcus type 3 antiserum (rabbit or horse) by the homologous polysaccharide diminished, in some cases to less than half. We can conclude that the precipitates contained correspondingly less antibody. The amount of antibody precipitated from rabbit antiovalbumin serum by ovalbumin, however, was relatively little affected. Heidelberger and co-workers have made use of this salt effect to dissociate antibody from pneumococcus precipitates (page 55).

Effect of *p*H. Mason (131) found that precipitin reactions occurred between *p*H 4.5 and 9.5; outside this range immune precipitates would dissolve. Within moderate *p*H ranges, say 6.6–8.0, the amount of precipitate is approximately constant (80, 81, 129).

Effect of Temperature. Although higher temperatures accelerate precipitation, the effect of temperature on the final amount of precipitate is generally small. Thus Heidelberger *et al.* (87) failed to find any influence of temperature on the amount of precipitate formed by mixing egg albumin and horse anti-egg antibody. In some cases (S3 and horse antipneumococcus type 3), however, Heidelberger and Kendall (78, 79) found that considerably less precipitate was formed at 37°C. than at 0°. The precipitate formed with the reagents in certain proportions could be dissolved by warming to 37°, or even to room temperature, and would reappear on cooling. Duncan (47) found a similar situation with yeast gum and an antiyeast serum.

These effects are evidently connected with the second, rather than the first, stage of antibody–antigen reactions, unless we are simply dealing with an effect of temperature on solubility.

Lipides. Lipides were found by Horsfall and Goodner (107) to form from 4 to 51% by weight of the precipitate formed with S1 and (horse and rabbit) antipneumococcus type 1 serum. Marrack and Smith (130) could not demonstrate any lipide in the precipitate made with horse serum pseudoglobulin and its antiserum.

Velocity of the Reaction. Zones and Optima

The reader will already have gathered that the velocity of precipitate formation is different in the different zones defined above. The practical use of velocity observations, in the form of the Ramon titration, and the Dean and Webb method of optimal proportions, has also been referred to in Chapter II, in the sections devoted to methods of measuring antibody concentration. The technic of carrying out such titrations is described in Chapter XI. We must now take up the question of the differences between these two procedures.

In the Ramon technic, varying amounts of antiserum are added to a constant amount of antigen (toxin), and the most rapidly flocculating mixture is observed. This has been called the β procedure by English writers (see 129). In the Dean and Webb technic, the amount of antiserum is held constant, and the amount of antigen is varied (α procedure). If another amount of antiserum is taken, and varying amounts of antigen again are used, it is found that the ratio, (dilution of antigen)/(dilution of antiserum), is the same, or nearly the same, as was obtained with the first amount. Thus in the α procedure, if most rapid flocculation occurs in the case of antiserum diluted 1:5 and antigen diluted 1:200, then we shall find with antiserum diluted 1:10 that the optimal dilution of antigen will be 1:400. This constant ratio of dilutions is referred to as the "optimal proportions" ratio. The question now arises, will the optimal proportions ratios obtained by α and β procedures be the same? It might possibly be expected that they would. A number of experimenters have tested this and have found that in general the two points are not the same (37, 48, 133, 170, 171, 172).

The proper examination of this point involves extensive "checkerboard" experiments, in which all practicable dilutions of serum are tested with all practicable dilutions of antigen. If this is done, a table of times of flocculation, one entry for each combination of antibody dilution and antigen dilution, will be obtained. The result of a typical experiment is shown in Figure 35.

In this experiment, the time of particulation, i.e., for particles to become just visible to the unaided eye of the operator, was recorded. The reactions were carried out with the tubes one-third immersed in water at 40°C. so as to obtain strong convection currents, which greatly accelerate the reaction, probably proportionally in all tubes (101).

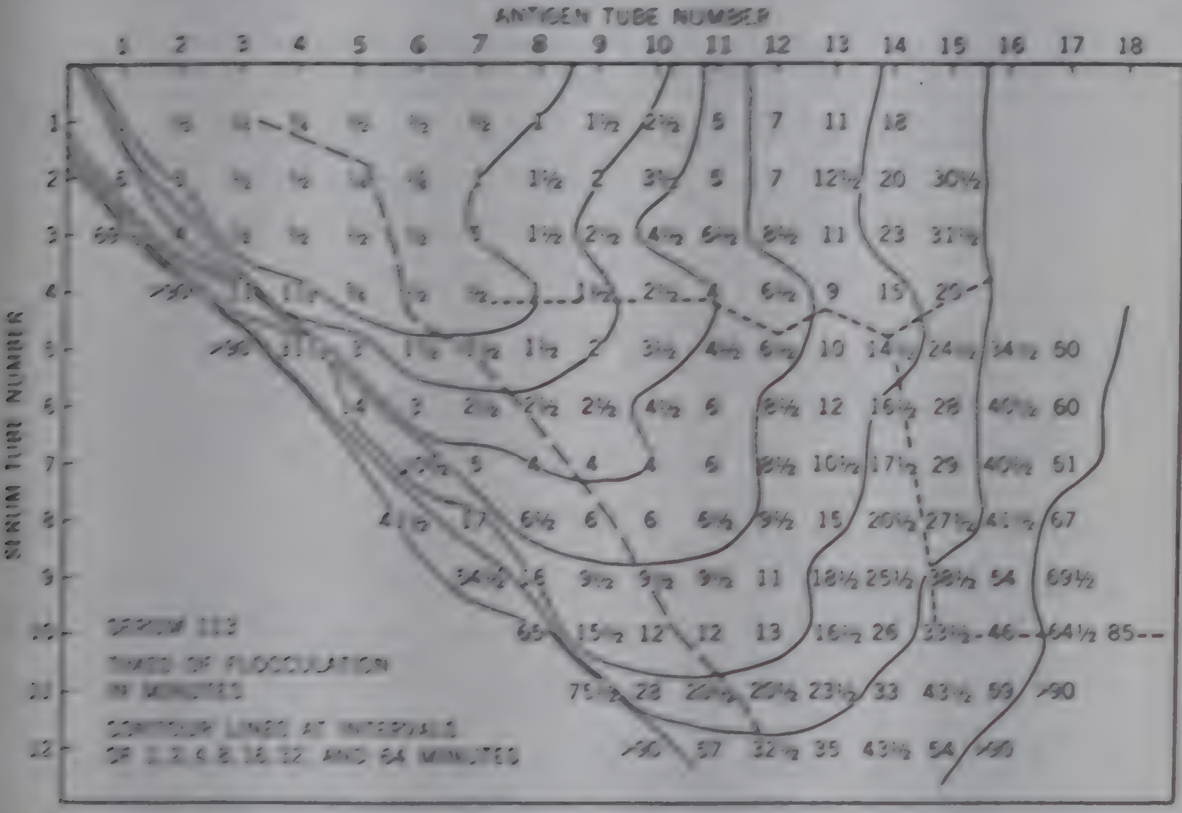
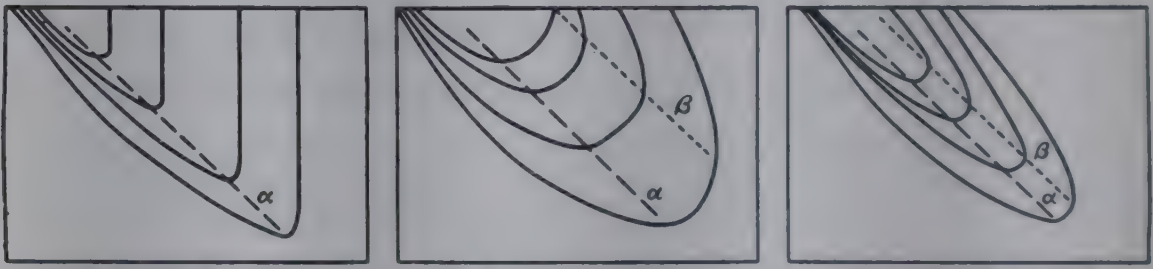


FIG. 35. Table of times of flocculation of mixtures of various dilutions of *Limulus hemocyanin* (top) and various dilutions of antiserum 113 (left), showing method of drawing lines of approximately equal times. Dilutions of antigen (reading left to right) and serum (reading down) successively 1.5 times greater (17).

The sort of result obtained in the α procedure can be found by following along one of the horizontal rows, where antigen concentration varies and antibody concentration remains the same. A minimum appears in the times recorded in this row. In the row above or the row below, there will also be a minimum; and the ratio of the concentrations (or dilutions) will in each case be the same, though in the lower rows the total time, since both reagents are more dilute, will be greater, even at the optimum.

The results which would have been obtained if the β procedure had been used are obtained by following vertically one of the columns. In the above experiment, in several of the columns at least, a minimum is also observed. However the ratio of the dilutions at the optimum is not as constant as it was in the case of the α procedure

Mathematically, the set of data shown in Figure 35 defines a surface, where time is the third dimension, and antigen dilution and serum dilution are the two independent variables. (Note that we are plotting these on a logarithmic scale; this is the result of giving simply the tube numbers instead of actual concentrations, but is in any case necessary in order to achieve sufficient compactness.) This surface could be modeled in three dimensions, and the model would summarize the flocculative behavior of the system. Such a model is not easy to show in two dimensions, but the essential characteristics can be shown by making use of conventions similar to those used in topographic maps (17). Lines can be drawn through points of equal time, at appropriate intervals, and the position and distance apart of these contour lines (isochrones) serve to characterize the surface. A number of such experiments were carried out by the present author (17).



(A) R TYPE (ONE OPTIMUM) (B) H TYPE (TWO OPTIMA) (C) EXTREME H TYPE (OPTIMA CLOSE TOGETHER)

Fig. 36. Ideal isochrones for antisera of various types (see text) (17).

The contour lines can not be drawn at equal intervals; therefore the intervals have been successively doubled. The line of dashes connects the minima obtained by the α procedure, while the line of dots connects those of the β procedure. For a constant "optimum" to be obtained by either method, the corresponding line should be straight, with a slope of 45°.

Classes of Antibodies. It has been suggested (17) that sera may be divided roughly into two classes, designated as R and H. It will perhaps make the distinction between these different types of antisera clearer if we present a set of ideal contour lines illustrating them, where the nonessential irregularities have been smoothed out. This is done in Figure 36, which shows the ideal behavior of the R type, the H type, and the extreme H type as typified by diphtheria antitoxin.

In the R type only one optimum, that obtained by the α procedure, is possible. In sera of the H type, two optima are possible, depending on the procedure, and it is clear from Figure 36 that these two optima can never actually coincide. See also Teorell (174). In the H system, the contour line passes through a minimum and then curves over to the left, so as to create another minimum under the conditions of constant antigen. This may be seen by turning Figure 36 clockwise through 90°, so that the right-hand side as viewed in the usual way becomes the bottom. In these sera, apparently, combination of more than a certain amount of antibody with a given amount of antigen does not leave the rate of flocculation at its maximum, but actually diminishes it. Duncan (13), Miles (133), and Taylor (170) have reported experiments in which this behavior of certain rabbit sera is even clearer.

Horse antitoxin of course furnishes the classical example of what is meant here by an H serum. In such sera (extreme H type), where the change in slope of the curves is extremely rapid near the point of inflection, the difference between the two optima will be slight. This has been observed in titrations of diphtheria antitoxin, where the optima are so close together that there is only a 10–15% difference between them (175). A number of workers, working with rabbit serum, have found well-marked optima with both the α and β procedure (47, 48, 170).

Relation of Flocculation Optima to Neutralization

Dean and Webb (44) found that at the optimum obtained by their (α) procedure, in the case of the system with which they worked (antibody = rabbit anti-horse serum, antigen = horse serum), there were no more than traces of antibody and antigen in the supernatant. Similar results have been obtained with ovalbumin, azoproteins, yeast gum, and pneumococcus polysaccharide type 1 (references in 17 and 129). This suggested that the flocculation optimum had in addition to its practical utility some theoretical significance.

The fact that the β optimum seems to correspond in at least some cases to some point in the equivalence zone may be accidental, but is more likely due to the fact that in both cases the deciding factor is approximately complete coverage of the surface of the antigen by antibody. This is conceivable on the basis of either the older or newer theories of reaction mechanism, as can be seen by the remarks of Pauling (150). The studies of Boyd and Hooker (24, 27) on the ratio of antibody to antigen in precipitates suggest that the antibody covers more surface than if it behaved as a sphere, and purely chemical and physical evidence suggests that antibody molecules are elongated ellipsoids (see page 45). In the case of ovalbumin, the antigen surface is calculated to be about completely covered by three molecules of antibody. The studies of Heidelberger (74) and collaborators suggest that the compound formed in this system at the antibody excess end of the equivalence zone is mostly A_3G (Table XXXIV).

In the case of diphtheria toxin, the β optimum and neutralization sometimes coincide. Pappenheimer and Robinson (147) failed to detect, even by the sensitive rabbit intracutaneous test, any appreciable toxin or antitoxin in the supernatant from such mixtures. Glenny (68) has however found considerable variations in precipitates of toxin and antitoxin in the most rapidly flocculating proportions, reporting some to be neutral, some overneutralized, and some underneutralized. Duncan (48) and Taylor, Adair, and Adair (172) found instances where the Ramon optimum was in the region of antibody excess. Burnet (32) and a number of others found most rapid flocculation of staphylococcal toxin and antitoxin in mixtures containing some excess of antibody. Malkiel and Boyd (127) found in two hemocyanin-antihemocyanin systems that most rapid flocculation occurred in mixtures where antigen was in excess.

Interpretation of Flocculation Optima

Attempts to account for the difference between the α and β optima will be found in papers by Brown (29), Boyd and Purnell (28), and Teorell (174). They are based chiefly on the assumption that different classes of antibodies and antibody-antigen compounds have different solubilities. Boyd and Purnell (28) also point out that the α and β optima can never really coincide, even theoretically, since the two procedures are essentially different.

Linear Relation of Velocity and Antigen Concentration

Hooker and Boyd (101) observed that, when there was a considerable excess of antibody [three to four times the (α) optimal amount], the times of flocculation varied inversely as the concentration of the antigen, so that on plotting them a straight line was obtained. This was interpreted in the light of von Smoluchowski's theory of flocculation rates, based on the kinetic theory. After a certain amount of (type R) antibody has been added, the maximal possible change in surface properties of the primary aggregates has been effected (see Figs. 2, 32); further combination of antibody should not affect the fraction of collisions resulting in union (ϵ in the equation of von Smoluchowski). Therefore the rate of flocculation, in this region, would depend solely on the number of centers of aggregation, which equals the number of antigen molecules added. If this number is cut to one-half, for instance, the time of flocculation will be doubled, as follows from von Smoluchowski's mathematical treatment (101, 174). Hooker and Boyd (101) were also able to show that this relation would still be expected to hold if stirring, as by convection currents, was used to accelerate the reactions.

This relation permits in some cases the rapid and accurate estimation of extremely small quantities of antigen. A measured portion is added to an excess of antibody, and the time of flocculation is determined. The concentration of antigen in the sample can be read off from a graph of times and concentrations, constructed by determinations of the time of flocculation of known amounts of antigen.

Effect of Changes in Conditions on Velocity

Electrolytes. Flocculation does not occur in salt-free mixtures of diphtheria toxin and antitoxin. Addition of electrolyte enables flocculation to take place, and at first the rate is accelerated by each increase in electrolyte concentration. Higher concentrations (164, 165) delay flocculation somewhat. For sodium chloride, sodium bromide, and sodium iodide, the optimal salt concentration was between 0.05 and 0.25 *M*. The effect of the higher concentrations may be connected with their "salting-out" effect on proteins, but it is perhaps more likely that it is due to the tendency of salts to dissociate antibody from antibody–antigen complexes (see page 55).

Hydrogen Ion Concentration. Schmidt (164, 165) found that diphtheria toxin and antitoxin would flocculate at roughly the same rate at any *p*H between about 5.5 and 9.0, the optimum appearing to be about *p*H 6. As the *p*H was increased above 9, or brought below 5.5, the velocity fell off very sharply. Typical results are shown in Figure 37. Examples of the effect of *p*H and electrolyte concentration on precipitation have also been given by Bukantz and de Gara (31).

Effect of Temperature. Like the rate of flocculation of some inorganic sols (52), the velocity of serological precipitation has a rather high temperature coefficient. The rate usually rises rapidly (double or even more for each 10° rise in temperature) up to 20°C., somewhat less rapidly up to 30°, and less rapidly after this. At 56° it may be even slower. Eagle states that the decrease in viscosity, acceleration of Brownian movement, and possible increased velocity of antibody–antigen combination do not suffice to account for the magnitude of the temperature effect on flocculation. The slowing with higher temperatures is probably at least in some cases connected with protein denaturation.

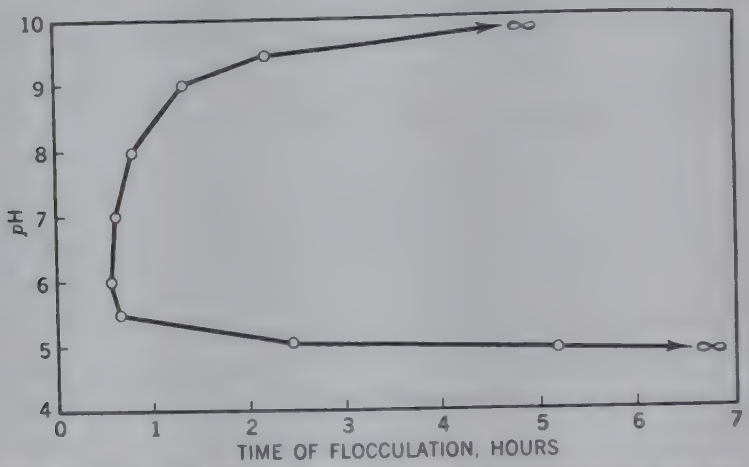


Fig. 37. Effect of *p*H on velocity of flocculation of toxin and antitoxin (164, 165).

Shaking, Stirring. Shaking and stirring markedly increase the velocity of flocculation, as they do agglutination. On the basis of von Smoluchowski's theoretical treatment, the accelerating effect should be proportional to the size of the particles. Therefore the effect on particles of the size of protein molecules is slight, being of the order of 0.1%, but the effect with larger aggregates is much greater. Eagle (52) has given an interesting illustration of this. He found that in the few seconds just after an antigen and antiserum are mixed shaking has little accelerating effect, but later, when larger aggregates are beginning to form, it accelerates powerfully, and may cut the time required for flocculation to less than half. The stirring due to convection currents also has a marked effect. The time of flocculation is greatly prolonged if convection is minimized, as by totally immersing the tubes in a constant temperature bath.

Lipides. Removal of lipides from antisera has generally been found to interfere with flocculation (references in 129). Horsfall and Goodner (106) demonstrated that removal of lipides from type 1 antipneumococcus horse serum caused a loss of the power of type-specific agglutination and precipitation, and in the case of rabbit serum a marked reduction in these activities. The initial activity could be restored to the horse serum by addition of lecithin, and to the rabbit serum by addition of cephalin. The amounts of these lipides required were small (0.1 milligrams per cubic centimeter of serum), even less than the lipoid content of the precipitates, which in these experiments varied from 4 to 51% by weight (107).

Nonspecific Serum Proteins. The effect of proteins other than antibody or antigen on the velocity of precipitation is probably rather variable, as it is in agglutination, but not many observations have been reported. Hooker and Boyd (105) found that mixtures of ovalbumin and antiovalbumin, containing normal rabbit serum instead of saline as a diluent, flocculated somewhat more slowly. It was not tested if this was simply due to the increased viscosity of the solution. Hooker (private communication) found that normal horse serum had a considerable retarding effect on the flocculation of *Busycon* hemocyanin and a horse antihemocyanin.

Other Substances in the Serum. It is not impossible that substances as yet unidentified may be found to affect the velocity of precipitation. Diphtheria antitoxin, when purified by precipitation with sodium sulfate, has been reported not to flocculate, although it still neutralizes (129). The possibility of some denaturation of the antibody has to be considered. Pappenheimer and Robinson (147) obtained flocculation with antitoxin purified by ammonium sulfate precipitation. It has been found (22, 100) that partially absorbed* sera, even in cases where the amount of antibody remaining is nearly as much as before, may show greatly increased flocculation times. These observations however could perhaps be explained by removal of lecithin or cephalin, a possibility that was not investigated.

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* Or "partially exhausted" (i.e., sera from which part of the antibody had been removed by precipitation with an amount of antigen insufficient to combine with all the antibody for a certain antigen).

Mechanism of the Precipitin Reaction

There have been several hypotheses proposed to account for the second stage of serological reactions in which precipitation or flocculation is produced. As yet there is not general agreement as to the correctness of any particular one of these, so it will be desirable to say something of each.

Theories of Arrhenius. Anxious to apply the law of mass action to immunological reactions, Arrhenius (7) tried to show that all the quantitative data then known could be accounted for on the basis of reactions obeying the laws of classical chemistry, without making use of the concepts of Ehrlich, which, although also chemical in nature, involved the assumption of the existence in the same system of a multiplicity of antibodies and antigens of different characteristics. The figures of Arrhenius show good numerical agreement with the experimental data, but his very success is now a lesson in caution, demonstrating again that numerical agreement between experiment and the predictions of a theory does not always show that the theory is correct. Since the composition of the antigen–antibody compound could not then be determined and since nothing was known of the molecular sizes of antibodies, antigens, or their reaction products, Arrhenius was able to make any assumptions he liked about the relative numbers of molecules taking part in the reactions and about the numbers of molecules of product formed. In the light of modern knowledge, most of these assumptions are seen to be in error. Thus, to account for the results of an experiment on the neutralization of ricin by antiricin, Arrhenius assumed that two molecules of antiricin reacted with two molecules of ricin to produce three molecules of innocuous substance. At that time little was known of the composition of the products of serological reactions, but today it is evident that this is an extremely improbable reaction.

Furthermore, in most cases, as in the antiricin–ricin reaction, Arrhenius did not consider the possibility that the reactants could combine in multiple proportions, although we now know this to be one of the outstanding characteristics of serological reactions.

In an attempt to account quantitatively for the inhibition zone of the precipitin reaction, Arrhenius did make the assumption that the reagents could combine in two different proportions, one of them giving a soluble compound. On this basis he was able to predict the *amounts* of precipitate observed relatively satisfactorily. From the data such as those cited in the section on the composition of the precipitate (page 243), however, it is clear that any predictions he might have made as to the composition of the precipitate would have been wrong.

The immunochemical career of Arrhenius illustrates the risks which are involved when a brilliant and successful worker in one field turns to work with another subject unfamiliar to him, particularly if he approaches the new subject without the spirit of humility proper to any novice.

Theories of Bordet. Perhaps the chief credit for insisting on the power of antibodies and antigens to combine in varying proportions goes to Bordet (13), although others had proposed the idea previously (see 74). Bordet stressed the similarity of serological to "adsorption" reactions, and went so far as to deny the applicability of the laws of ordinary chemical union to serological reactions.

We now know that there is no such fundamental distinction between adsorption and ordinary chemical reactions as Bordet thought (see page 240). Practically any reversible chemical reaction taking place at a surface where there are many reactive groups will follow a course similar to that of adsorption reactions (see 61). Consequently this difference between the views of Bordet and of Ehrlich or Arrhenius has reduced simply to the insistence of the former on combination in multiple proportions.

It was Bordet who insisted on the division of serological reactions into two stages, which still seems convenient. He pointed out the importance of salts in the reaction. He looked upon agglutination and precipitation as fundamentally analogous, and in the section on precipitation in his book (13) spoke of the formation of a layer of antibody over the surface of a particle, rendering it susceptible to the flocculating influence of electrolytes.

In some respects, at least, the theory of Bordet needs modifications. For example, he apparently thought of the layer of antibody as a thin film spread over the surface of the antigen, but we have seen (page 238) that there are reasons for believing that the film is not much thinner than a layer of antibody molecules, and therefore not strictly a "film." This again was probably the result of his overreliance on the adsorption analogy. It should also be mentioned that certain simple chemical compounds have been found to precipitate with antibody (18, 122, 152, 155), and in these cases there is some question as to the possibility of the formation of a film of antibody over their surface. This question, however, is not quite as simple as it seems at first sight, and will be discussed on page 267.

The idea of Bordet, briefly, was that antibody combined, in varying proportions which depended on the proportion in which the reagents were mixed, with the surface of the antigen, and in some way altered the surface properties so that flocculation could take place (see Fig. 2, page 12). He pointed out that the properties of the complex would vary with the proportion of the two reagents, and we could thus account for the inhibition zone with excess antigen. He thought of the second stage as nonspecific; it was of the "domaine de la physicochemie."

Recent work (see page 265) has in some cases suggested that the second stage is perhaps not always completely nonspecific, and that a certain degree of specificity, of how high an order is not known, may play a role under some circumstances. In this respect, therefore, the Bordet picture may prove to be somewhat inadequate.

Marrack (129) has extended the Bordet theory slightly: "When the antibody molecules are attached to the antigen, the polar groups, on which the solubility of the antibody globulin normally depends, are brought into apposition with each other, and attract each other instead of water molecules. . . . Only the polar groups on the free surface are left available for binding water. If these are insufficient to keep the complex in solution, the complexes will aggregate if the surface potential is below a critical level."

If we incorporate this slight emendation, the Bordet theory still seems capable of interpreting in a satisfactory manner most of the facts as we know them, with the above-mentioned exception of whatever degree of specificity is observed in the second stage, and perhaps of the precipitability of certain simple chemical compounds (haptens). The quantitative theory of Teorell (173) is based on assumptions essentially the same as Bordet's.

The "Alteration" = "Lattice" = "Mutual Multivalence" = "Framework" Theory. Marrack (129) pointed out that if the antibody has more than one combining group it might be possible for antibody and antigen to be bound together in the form of a coarse "lattice," which is shown diagrammatically in Figure 38. This involves a new concept, in that the aggregation is ascribed not to a loss of attraction for water, but to specific attraction between the particles. "This mutual attraction is due to the link provided by further antigen molecules" (129). Marrack apparently introduced this concept in order to avoid the supposition that the antibody after it has combined with antigen is denatured, although this idea does not seem to form a necessary part of the Bordet theory.

A very similar theory was developed by Heidelberger and Kendall (74), and later taken up by Pauling (150) and various other workers. Since the various proponents of the theory do not agree on a name,

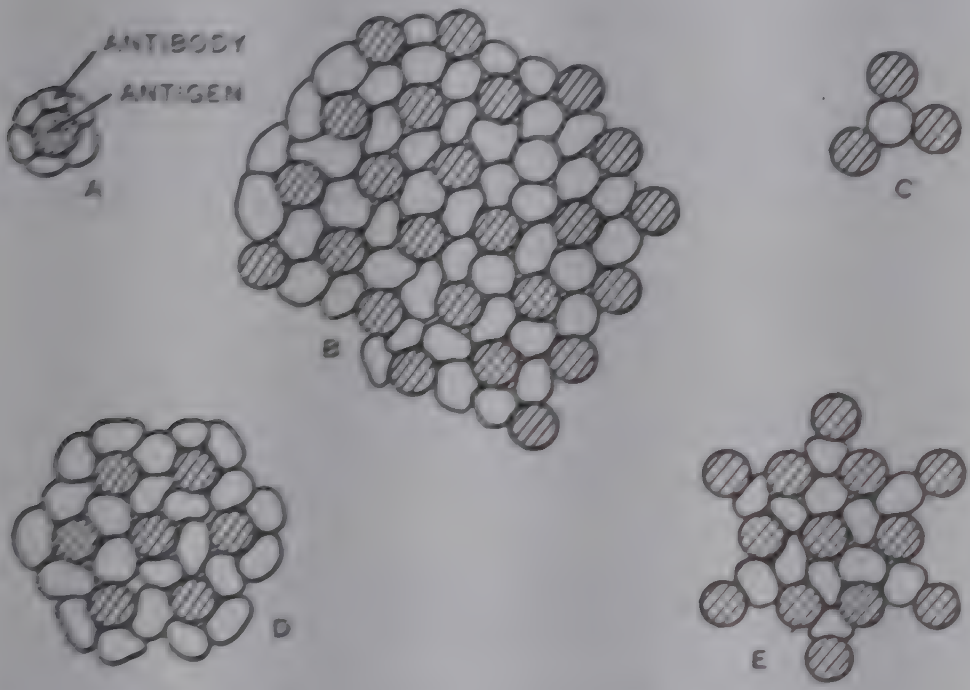


Fig. 32. Diagrams of hypothetical arrangements of antigen (shaded) and antibody (white) molecules in antibody-antigen complex (129): (A) primary aggregate, (B) complex structure at optimal proportions, (C) antigen excess, (D) antibody excess, (E) antigen excess. Such aggregates are not to be supposed to possess a structure as regular as that of a crystal lattice, but are to "be compared rather with a glass such as silica glass, in which each silicon atom is surrounded tetrahedrally by four oxygen atoms and each oxygen atom is bonded to two silicon atoms, but which lacks further orderliness of arrangement" (150).

and since all versions of it agree in postulating as essential for the formation of precipitates (and "agglutinates") an alternate interposition of molecules of antibody and molecules (or particles) of antigen, the term "alternation hypothesis" has been proposed (104). Probably at present the majority of American and British immunologists subscribe to this theory, although some feel that the theories of Bordet also contain much that is plausible. Workers on the

Continent do not seem to have taken so kindly to the alternation theory (71, 174).

Heidelberger's concept of the mechanism of the precipitin reaction (74) is based on certain assumptions and simplifications; in nearly his own words, they are: (*1*) Antigen (G) and antibody (A) are chemically and immunologically multivalent with respect to each other; that is, each substance possesses two or more groupings capable of reacting with the other. (*2*) The antibody may be treated mathematically as if its average behavior were that of a single substance. (*3*) The reaction is considered as a series of successive bimolecular reactions which take place before precipitation occurs. (*4*) The mass law applies, so that the rates of formation of the reaction products are proportional to the concentrations of the reacting substances.

The first reaction postulated is:

$$\mathrm{G + A \rightleftharpoons GA} \tag{2}$$

followed, for example, in the region of excess antibody, by competing bimolecular reactions due to the mutual multivalence of the components:

$$\begin{aligned} \mathrm{GA + A} &\rightleftharpoons \mathrm{GA{\cdot}A} \\ \mathrm{GA + GA} &\rightleftharpoons \mathrm{GA{\cdot}GA} \end{aligned} \tag{3}$$

A third step follows, in which the competing bimolecular reactions would be:

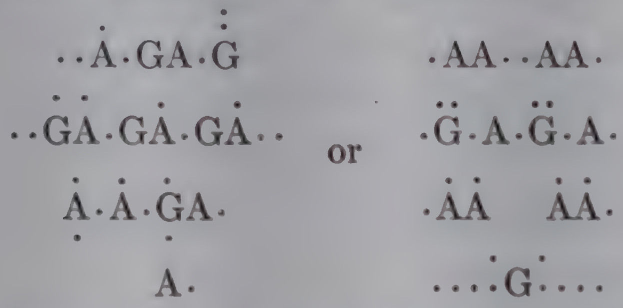
$$\begin{aligned} \mathrm{GA{\cdot}A + A} &\rightleftharpoons \mathrm{GA{\cdot}A{\cdot}A}, \\ \mathrm{GA{\cdot}G + A} &\rightleftharpoons \mathrm{GA{\cdot}GA{\cdot}A}, \\ \mathrm{GA{\cdot}A + GA{\cdot}A} &\rightleftharpoons \mathrm{GAA{\cdot}GAA}, \\ \mathrm{GA{\cdot}A + GA{\cdot}GA} &\rightleftharpoons \mathrm{GAA{\cdot}GA{\cdot}GA}, \text{ and} \\ \mathrm{GA{\cdot}GA + GA{\cdot}GA} &\rightleftharpoons \mathrm{GAGA{\cdot}GAGA} \end{aligned} \tag{4}$$

in which the first two reactions are supposed to occur only in the presence of enough A to carry the composition of the reaction product beyond the $\mathrm{GA_2}$ stage. Similarly, each compound formed in the third step would react with each other compound, or with more A, if present, to form still more complex substances, and the reaction is supposed to continue until particles are formed large enough to settle from the solution. Precipitation would take place at this point, "doubtless facilitated by the mutual discharge, with loss of affinity for water, of ionized or polar groupings brought together by the series of chemical reactions."

If A and G are mixed in equivalent proportions the reaction is supposed to consist simply of polymerization of the GA formed in reaction (2), in steps (3), (4), etc. The precipitate would be $(GA)_u$.

For the region of excess G similar expressions are proposed if G and A are interchanged in (3), (4), etc. In the presence of a large excess of G (in the inhibition zone) a soluble compound, G_xA, containing one more molecule of G in combination than the last insoluble compound, is supposed to be present in solution.

The final precipitate in each case is supposed to consist of antibody molecules held together in three dimensions by antigen molecules, which Heidelberger represents two dimensionally as:



"The process of aggregation as well as the initial hapten–antibody combination is considered to be a chemical reaction between definite molecular groupings."

Marrack has written as if he considers serological reactions essentially irreversible (see page 267), but Heidelberger merely says (74) in presenting the above theory: "Since no evidence of dissociation could be found over a large part of the reaction range, the equilibria postulated evidently lie very far to the right."

Teorell (173, 174) in the theory referred to above, in which he assumes antibody behaves as if it were univalent, assumes that the reaction of A and G results in the establishment of the following simultaneous equilibria:

$$A + G \rightleftharpoons AG, \text{ with dissociation constant } K_n$$
$$A + AG \rightleftharpoons A_2G, \text{ with dissociation constant } K_{n-1}$$
$$A + A_2G \rightleftharpoons A_3G, \text{ with dissociation constant } K_{n-2}$$
$$A + N_{n-1}G \rightleftharpoons A_nG, \text{ with dissociation constant } K_1$$

Precipitation is ascribed simply to insolubility of compounds such as AG or A_2G, and not to a "lattice" formation. Teorell further assumes that the mass law applies, even to the insoluble compounds, since he supposes that the combining groups of the antigens and antibodies composing them are still freely accessible.

The "Occlusion Theory." Boyd (18) offered an alternative theory of the precipitin reaction (which by implication would apply to other serological reactions), which he called the "occlusion theory." This attempts to explain the initiation of precipitation (or agglutination) as due to the lowering of the solubility (in the 0.15 *M* sodium chloride in which all these reactions are carried out) of the antibody–antigen compound due to mutual neutralization of polar (i.e., solubilizing) groups of antibody and antigen and concomitant steric hindrance of other polar groups of antibody molecules, owing to their being brought into such close apposition. That is, the molecules of antibody, when in combination with a molecule of antigen, are supposed to be so near each other that they literally get into each others' way, and many of their polar groups are nonspecifically turned aside from their business of attracting water (104), with the result that as a whole the antibody–antigen complex does not have enough free polar groups to keep it in solution. Kleczkowski (115a) evidently holds a very similar theory.

The whole subject of the mechanism of serological reactions is intricate and difficult. The present writer happens to be particularly interested in it, but it is hardly to be expected that all the readers of this book will be equally interested. For this reason, and because the question must at the present time be regarded as an unsettled one, it has seemed best to relegate a discussion of the relative merits of the Bordet, "alteration," and "occlusion" hypotheses to an appendix to this chapter.

Flocculation Tests for Syphilis

If the blood of syphilitic patients contained antibodies for the spirochete, it ought to be possible to demonstrate them by the precipitin test, using a suitable extract of the organisms which might contain specific proteins, carbohydrates, or possibly lipide (116). But one serious difficulty immediately presents itself: according to several authorities (56, 187), no strain of *Treponema pallidum* yet cultivated is pathogenic, and the latter authors (187) state that there is no relation, serologically and immunologically, between the virulent and the cultured organisms. They suggest that the cultivated organism has, in the language of the laboratory, gone "rough" (lost a surface antigen), and such organisms would not be expected to furnish specific extracts.

Nevertheless it is possible to test serologically for syphilis, and such procedures have much clinical importance. The antigens first used for such tests were made from organs of syphilitic human beings, but it was soon found by Landsteiner that extracts of normal organs, even from animals would do. It is now known that these active extracts consist of various normal lipides. At first this seemed to prove that the reactions obtained with the bloods of syphilitic patients were biologically nonspecific, but clinical experience has shown that on the contrary they are practically specific for syphilis. It is now thought, at least by some workers (56) that there is a real serological relationship between some antigen in the spirochete and some of these lipides. This is supported by the observation that absorption of syphilitic sera with washed spirochete suspensions (of certain strains) removes the "reagin" for these lipide-containing extracts.

At the same time that it was considered that the "antigens" used in the serological tests for syphilis had nothing to do with the spirochete, it was doubted if antibody ("reagin") in the patient's serum responsible for the reaction bore any relation to antibodies against the spirochete. The observations of Eagle and Hogan (58) however, tend to support the thesis that the serum change in syphilis is primarily an antibody response to *T. pallidum*. The reactivity which these antibodies display for the tissue extracts are, according to these workers, due to a serological similarity between some of the normal tissue lipides and antigens of the spirochete. (This recalls the observations on the Forssman antigen; see Chapter IV.) In support of this contention they cite the above observation on the absorption of syphilitic serum with spirochetes, and also the very suggestive observation that immunization of rabbits with tissue lipides will produce agglutinins and complement-fixing antibodies for the spirochetes (see 121). Other workers (see 179), however, do not support this view that the Wassermann antigen is spirochetal in origin. Weil (179) seems to think the Wassermann antigen may come from either the spirochetes or the host's own tissues.

Having, however, an antibody of some sort to the spirochete in syphilitic serum, and having available a serologically related antigen in the form of the lipide-containing tissue extracts used, it might be possible to observe a specific precipitation reaction between them.

If we accept the above notions, this is apparently essentially what the flocculation tests for syphilis consist in. A suitable preparation of the lipide-containing tissue extracts is mixed with serum from the patient, and in positive cases a flocculation or precipitation is obtained (Sachs-Georgi, Kahn, Eagle, etc.). The extract generally used is an alcoholic extract of beef heart, to which cholesterol is usually added. According to Eagle (53, 54), the function of the cholesterol is to serve as centers of adsorption for the tissue lipides, so that the particles of "antigen" will be larger, and hence more suitable for precipitation (and complement fixation), when the mixture is added to water. See page 295.

Examination of purified syphilitic reagin indicates that it is associated with two components with sedimentation constants of 7 and 19 Svedbergs, and has an electrophoretic mobility between that of of the β and γ globulins (113).

The mechanism of reaction is supposed (53, 54) to consist in the combination of the protein reagin (or antibody) from the serum of the patient with the particles of lipide-coated cholesterol, thus sensitizing the particles to agglutination by the electrolyte present. The reaction is therefore in all respects similar to other antibody–antigen reactions.

Various hypotheses of the Wassermann reaction are discussed by Weil (179). The Wassermann reaction itself will be discussed in the next chapter.

C. Appendix: Hypothetical Mechanisms of Serological Reactions

1. Agglutination

Before we seek an explanation of the agglutination phenomenon, we ought perhaps to inquire how much needs explaining. Bacterial and other cells have always a certain attraction for each other, and an aqueous suspension can only be obtained if the attraction for the water is greater, or is made greater, than their mutual attraction. Their mutual attraction can be overcome by repulsion forces due to electric charges on the cells. Under the microscope the cells in an erythrocyte suspension are seen not to be distributed in truly random fashion, but to be at almost exactly equal distances from each other, almost certainly held apart by electric repulsion. The first sign of incipient agglutination is often a modification of this equidistant arrangement in the direction of a haphazard arrangement. Acid-fast bacteria, owing to their lipide nature, do not form hydrophilic suspensions. With other bacteria it is not always easy to obtain uniform stable suspensions, and some kinds of bacteria often spontaneously agglutinate, particularly in low salt concentrations; (here there can be no question of "framework" formation). It has been reported (136) that salt-free erythrocytes (if prevented from hemolysing by the presence of sugar), are agglutinated readily by very small quantities of electrolyte. Many workers have observed (see 129) that bacteria, like other particulate suspensions, agglutinate when the surface potential falls below about ± 15 mv. The mutual attraction of bacteria decreases with rising salt concentration as was shown by Northrop and de Kruif (143, 144) by direct measurement, and it is found that higher salt concentrations favor stable suspensions. Thus it is evident that in most cases it would not require any very great change to transform a stable bacterial suspension to an unstable one which would then spontaneously agglutinate. A slight alteration would do (see Dubos, 46, page 55). Neisser and Friedemann (144) observed that bacteria which had been treated with lead acetate and subsequently washed free of all soluble lead were promptly agglutinated upon treatment with hydrogen sulfide. The antibody-like action of tannin, which agglutinates erythrocytes (and sensitizes them to lysis by complement) and detoxifies diphtheria and tetanus toxins, also seems hardly to be serologically specific, but more likely seems to be due to physicochemical modifications of the surface of the cell or molecule subjected to its action (62). The question that remains is: through what mechanism does the deposition of antibody on the surface cause the bacteria to agglutinate?

Most bacteria are strongly electronegative at reactions approaching neutrality; the usual result of combination with serum (sensitization) is a reduction of the potential difference (138). Thus the surface potential of all sensitized bacteria tends to approach the same level, which is that of serum globulin at the same *p*H and salt concentration. Another and more important alteration in the surface is the transformation of its hydrophilic character to a more or less hydrophobic character, even in moderately high salt concentration. The cohesive force between bacteria is found to be increased by their combination with agglutinin, roughly proportionally to the amount of agglutinin used (142). It can readily be imagined that changes such as these could account for the agglutination which follows combination with antibody, as was believed by Bordet (13, 14).

Motion pictures taken with the aid of extremely brilliant illumination (sunlight), and electron microscope pictures of bacterial agglutination give some information as to the probable mechanism (157–159). At least three kinds of bacterial agglutination seem to be possible with certain (flagellated) bacteria. Flagellar (H) agglutination seems to be the result of the deposition of agglutinin on the flagella in the form of a film of antibody molecules radially disposed (standing on end) (5, 137). The flagella become immobilized, and later entangled mechanically. This results in agglutination (157, 158).

In the case of somatic (O) agglutination, the antibody seems to combine with the body of the cell, and such "sensitized" bacteria no longer repel, but become attracted towards each other. Pijper (157a) says: "It is tempting to think of changes in electrical charge." In "Vi" agglutination Pijper (158) believes three factors operate: an increasing paresis of the motile organisms, continued normal repulsion between bacilli, and "stickiness" of the surface of the bacteria, which results in their sticking together when they happen to collide (see Dubos, 46).

Landsteiner (personal communication) long ago observed that precipitates and agglutinates sometimes exhibit a tendency to stick to the walls of the test tube, as if they were nonspecifically sticky (see 98). Erythrocytes agglutinated by the globulins of certain beans (see page 40) also often exhibit this phenomenon, even when the agglutination is rather specific (15). This would seem to make it extremely likely that primary aggregates may often stick together without the action of serologically specific forces, and we therefore ought to allow for this in speculating on possible mechanisms for the formation of aggregates.

If these observations are correct, the Bordet explanation seems most probable for the O type of agglutination, and the Marrack-Heidelberger explanation for the Vi type. Landsteiner (120) thought that it might turn out that both mechanisms operate in serological reactions.

The chief difference in observable phenomena predicted by the two theories is that according to the newer theory the agglutination of one kind of cell ought to be entirely independent of the agglutination of another serologically unrelated cell, since the aggregation is specific throughout, i.e., there is supposed to be no difference in the mechanism underlying the two stages of the reactions, combination and aggregation. There are two ways of testing this. One may mix two systems (two independent cell–antibody mixtures) and observe whether the clumps contain only one, or both, kinds of cells; or one may mix two systems and observe effects on the velocity of agglutination. On the basis of the alternation theory, the velocity of each agglutination should remain the same (or possibly be slightly decreased because of the interference caused by the presence of the other reagents) for the two systems cannot "cross-react," that is, aggregates cannot be formed by the collision of sensitized cell A with sensitized cell B, and so on. It has been assumed that the prediction of the Bordet theory, if antibody is assumed to be univalent, ought to be that the velocity, in the case of systems progressing at the same rate, should be just double for the mixed system, and proportionally different in the case of originally unequal velocities, and Hooker and Boyd (103) predicted and observed this in the case of mixed precipitating systems.

The results of mixing independent agglutinating systems has been tested by a number of workers (2, 3, 103, 177, 180). Mixed aggregates have sometimes been observed, especially with concentrated antibody, and the velocity of precipitation in mixed systems has sometimes been found to be increased (103); but, on the whole, when antibody is not present in excess, the aggregates tend to be homogeneous, which agrees with the prediction of the alternation theory.

Pressman, Campbell, and Pauling (161) have reported experiments in which erythrocytes coupled with not over 60 azo groups per cell were specifically agglutinated by antibodies for the attached groups. They calculate that, even if the antibody were spread out to a layer only 3.5 Å. thick, the 60 molecules of it would cover only 0.02% of the cell surface. They are therefore inclined to doubt if the agglutination observed by them is due to changes in the surface properties of the cells. It is mentioned by Dubos (46), however, that gelatin and edestin can affect the stability of suspensions of *Escherichia coli* in such great dilutions that it seems only localized patches of the cell can be covered.

It is apparent that the available evidence is conflicting. There is no doubt that the recorded observations are reliable; they simply do not agree in supporting wholly the Bordet or the alternation hypothesis.

We may mention certain observations made in the study of agglutination which are hard to explain solely on the basis of the alternation hypothesis. Experiments with agglutinating sera showing prezones, described on page 228, seem to show that the combination of the antibody with the bacterial surface is a different thing from agglutination, and that combination can be complete before agglutination begins, as in the experiments of Jones and Orcutt (112), in which unagglutinated cells from the prezone were induced to agglutinate by washing. These workers apparently demonstrated, by direct measurement, that agglutination is simply a result of the increased cohesiveness due to combination of antibody with the bacterial surface, for, when such cohesiveness was not produced (due to the presence of the inhibiting substance), no agglutination followed, even though plenty of extra antibody was present to provide "links."

Since the first stage of serological reactions is very rapid, a particle of antigen exposed to the action of an excess of antibody is probably maximally coated within a few seconds or minutes. Such coated particles, according to the "lattice" theory, should not be able to combine, as there would be no free combining groups (of a sort usable by the antibody in question) left; for, according to this view, a molecule or particle of antigen coated with antibody would combine only with similarly coated particles through the mediation of molecules of free antigen, and, in the absence of free antigenic groups, could not combine with a particle of antigen completely coated with antibody. Nevertheless, Boyd and Hooker (26) found that red cells, when agitated (after prolonged exposure to excess of agglutinin and, therefore, maximally coated), promptly and firmly agglutinated in the usual way. This seems possibly to be a case of agglutination due to nonspecific forces, unless after mixing the coated cells there is time for antibody to dissociate, exposing combining sites on the red cells which could then combine with free groups of antibody molecules on other cells.

2. Mechanism of the Precipitin Reaction

The Bordet Theory

The Bordet interpretation of the precipitin reaction did not differ in any essential respect from his interpretation of agglutination, which has already been discussed in the previous section. The quantitative theories of Teorell (173, 174), which are based on much the same sort of assumptions, were presented on page 259.

The "Alternation" Theory

Marrack (129) states: "If only two determinant groups are attached to one molecule an aggregate of unlimited size (AGAGAG—) can be built up," and thus seems to expect lattice, and therefore precipitate, formation even with a simple hapten, provided it contains two reactive groups in its molecule.

Heidelberger (74) says: "The process of aggregation as well as the initial hapten–antibody combination is considered to be a chemical reaction between definite molecular groupings. . .the entire course of these instances of specific bacterial agglutination could be accounted for. . . ." He thus seems to claim that larger aggregates are formed solely by the specific linkage of antibody groups with antigen groups.

A number of experiments have been made to test Marrack's assumption (18, 86, 98a, 104, 154). Some divalent haptens have failed to precipitate with antibody, but others have been observed to precipitate. Since such haptens, like many if not most organic dyes, are mostly aggregated in aqueous solution, the test is not strictly a rigorous one (21), unless a hapten which can be shown to be molecularly dispersed under the conditions studied can be induced to precipitate. For aggregates of molecules of a divalent hapten would possess many combining groups, say 20 or more (21), and would thus not be expected to behave very differently from ordinary antigens. The aggregation and resulting colloidal character were thought by Landsteiner and van der Scheer (121), who first observed specific precipitation of simple synthetic substances by antibody, to be the most probable explanation, especially as they found that precipitation was better if the hapten solutions were allowed to "ripen" (stand for a few days) before being tested. Other observations indicate a general sort of correlation between the degree of aggregation of a hapten and its precipitability by antibody (15). Also the solubility of the compounds has an important effect (18, 55). On the other hand, we must record the fact that no hapten containing only one combining group for antibody has yet been observed to precipitate. This may be considered as on the whole in favor of the alternation hypothesis.

To explain observed phenomena, proponents of the alternation theory have sometimes made assumptions which seem unnecessary. Hooker and Boyd (101) found that the linear relation observed between the dilution of the antigen and the time of flocculation (page 251) could be accounted for satisfactorily, in Marrack's words (129), by ". . . the reduction in the number of antigen molecules which serve as centers of aggregation" and that the additional assumption made by Marrack to account for the slower aggregation in the region of antibody excess "... because the formation of a continuous lattice fails progressively more and more as no further antigen molecules are available to provide links for the formation of larger structures when aggregates of moderate size ... are formed" was totally unnecessary. Actually, even with any practicable excess of (rabbit) antibody, larger structures usually *are* formed and become visible at the time predicted by the nonspecific hypothesis. This suggests that this particular aspect of the subject (the rate of flocculation) could be dealt with on the basis of the Bordet hypothesis alone (see 173, 174).

Mathematical Arguments

Quantitative theories of the precipitin reaction have been developed by various workers on the basis of the alternation theory (7, 90, 91, 115). Equations derived from them have been observed to fit the data obtained from the study of certain parts of the range of antibody–antigen combinations. However, arbitrary equations (80, 81) and various adsorption equations often fit equally well, as can be seen from the following examples.

The quantitative course of serological reactions was first studied mathematically by Arrhenius. Being a chemist, he naturally assumed that the mass law was applicable. In its simplest form, however,

$$\frac{\text{(free toxin)(free antitoxin)}}{\text{(toxin–antitoxin compound)}} = K$$

(the parentheses indicate concentrations of the respective substances and K is a constant), this formula does not apply, so Arrhenius assumed that the reaction took place according to the equation:

$$\text{toxin} + \text{antitoxin} \rightleftharpoons 2\ \text{(toxin–antitoxin)},$$

that is, that the reaction of one molecule of antitoxin with one of toxin gave two molecules of product. This led to the following relation:

$$\frac{\text{(free toxin)(free antitoxin)}}{\text{(toxin–antitoxin)}^2} = K$$

The values of q shown in Table XXXVI were calculated from this. Arrhenius considered that the numerical agreement proved that the assumptions made were correct, but his formulation is really in error in two respects. Toxin and antitoxin combine in more than one proportion, instead of giving only a single type of compound as Arrhenius assumed, and a molecule of toxin and one of antitoxin give only one, not two molecules of product. The agreement between the observed and Arrhenius' calculated values of q is therefore fortuitous. Since toxins and antitoxins combine in multiple proportions, the above simple mass law expression is not applicable, and we must assume a whole set of equilibria (19, 173, 174).

TABLE XXXVI

OBSERVED TOXICITY (q) OF TETANOLYSIN AFTER ADDITION OF ANTILYSIN, SHOWING ALSO VALUES OF q CALCULATED IN VARIOUS WAYS

Antilysin (cc.)	q (Observed)	q (Calculated by mass law)[a]	q (Calculated by "adsorption")[b]	q (Calculated by "adsorption")[c]
0	100	100	100	100
0.05	82	82	82	83
0.1	70	66	65	67
0.15	52	52	50	52
0.2	36	38	37	39
0.3	22	23	21	22
0.4	14.2	13.9	13.4	14.1
0.5	10.1	10.4	9.5	10
0.7	6.1	6.3	5.3	6.1
1.0	4.0	4.0	3.7	3.8
1.3	2.7	2.9	2.7	2.8
1.6	2.0	2.5	2.1	2.1
2.0	1.8	1.9	1.6	1.7

[a] Equation of Arrhenius.
[b] Equation of Langmuir
[c] Equation of Ghosh.

By making various assumptions about the dissociation constants in such equilibria, different results are obtained. Boyd (19) pointed out that assuming all the dissociation constants equal gave the Langmuir "adsorption" equation:

$$R = nks/(1 + kx)$$

where R is the amount of antibody combining with a fixed amount of antigen (or vice versa), n and k are constants, and x is the final concentration.

Ghosh (65, 66) derived an equation for antibody–antigen reactions, different in appearance, but algebraically identical with the equation of Langmuir. He demonstrated that it will fit the results of quite a variety of neutralization experiments. The sort of fit between observations and calculations obtainable with the earlier erroneous equation of Arrhenius and with this more plausible formula, is shown in Table XXXVI. (The slight differences seen there between the results predicted from the Langmuir and the Ghosh equations are due to slight differences in the choice of constants, and perhaps some slight errors in calculation.)

Heidelberger and Kendall (80, 81) predicted a linear relation between the ratio of antibody to antigen (R) and the amount of antigen nitrogen precipitated (with a constant amount of serum). This relation may be represented as:

$$R = a - bG \tag{5}$$

where R is the ratio of antibody to antigen in the precipitate, G is the milligrams of antigen nitrogen precipitated, and a and b are constants. Since in this region all (or nearly all) of the antigen is precipitated, in most of the range studied this is equivalent to a linear relation between R and the amount of antigen added, thus:

$$R = a - bG_a$$

This is illustrated in Figure 39, where the solid lines represent the Heidelberger-Kendall relation, and the circles are the experimental points.

Farther in the region of antibody excess, the linear relation does not hold, and this is seen to be true from Figure 39, in which the curved dotted lines show how the addition of a second (squared) term to the equations of Heidelberger and Kendall improves the agreement with the data (see 156). Heidelberger and Kendall (80, 81) themselves found that better agreement was obtained by plotting, not equation (5), but the curvilinear relation:

$$R = a - bG_a^{\frac{1}{2}}$$

In Table XXXVII will be found data on the composition of precipitates in the region of antibody excess and an example of the fitting of experimental data by the two equations of Heidelberger and Kendall and by equations of the "adsorption" type. All predict the composition of the precipitate moderately well. We may note that application of the "least squares" analysis indicates that in the present case definitely the best fit is obtained with the "Freundlich" type of equation.

The suitability of the others decreases in the order: Heidelberger and Kendall's curvilinear equation, their straight line equation, and

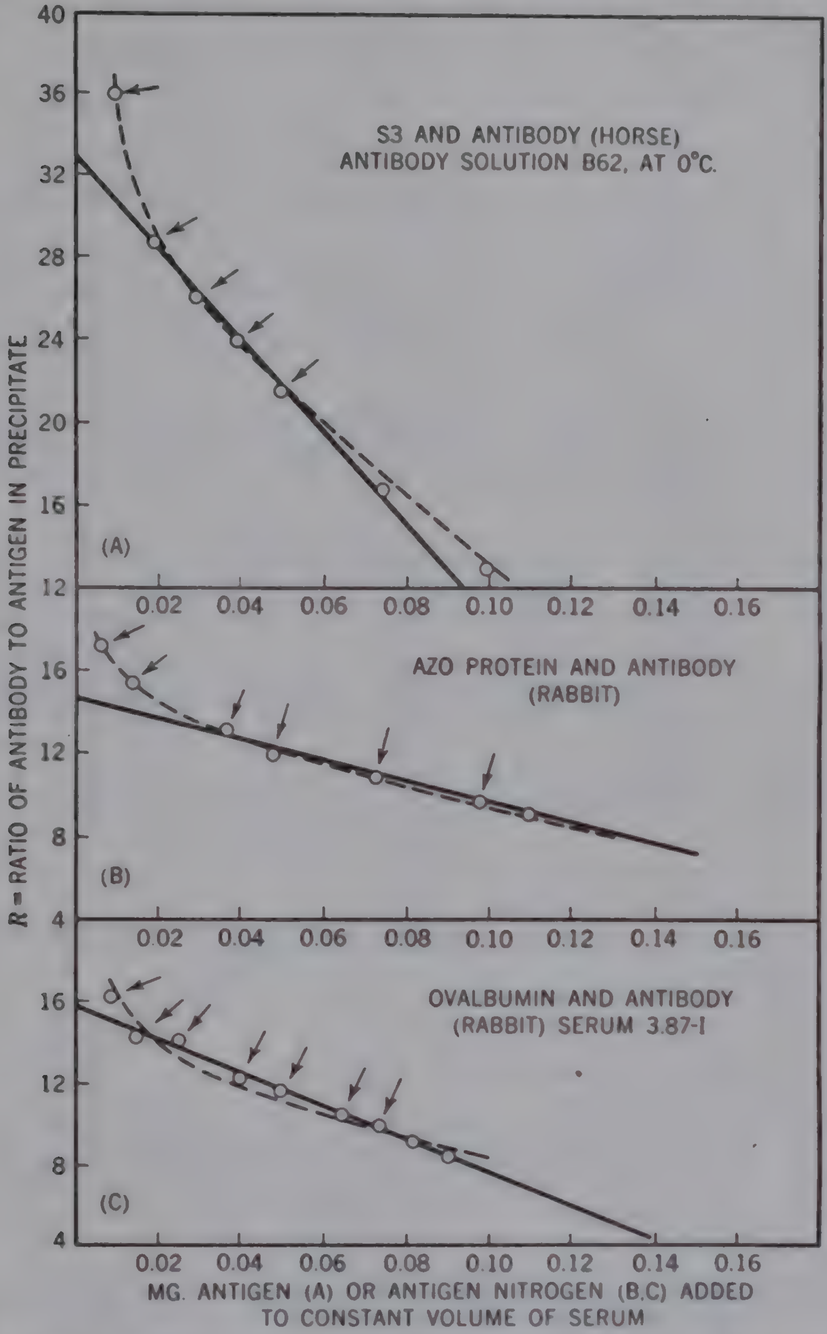


Fig. 39. Data showing relation between composition of precipitate and amount of antigen added to unit of antibody, with curves showing fitting by Heidelberger and Kendall's linear relation (solid lines) and a curvilinear (Boyd) relation (dashes). Arrows indicate points in region of antibody excess, to which the Heidelberger-Kendall relation (80, 81) was supposed to be particularly applicable. (Compare Figures 25 in reference 129.)

the Langmuir-Ghosh equation. The fit obtained with the Freundlich equation is about as good as could be expected from the data. Other sets of data might be expected to conform better in some cases to one of the other equations.

For the region of antigen excess, Heidelberger and Kendall proposed a different equation:

$$G = k - k'/G_a$$

TABLE XXXVII

QUANTITATIVE RELATIONS BETWEEN AMOUNT OF (*Brucella*) ANTIGEN ADDED, AMOUNT OF ANTIGEN AND ANTIBODY PRECIPITATED, AND COMPOSITION OF PRECIPITATE,[a] WITH PREDICTED RESULTS FROM VARIOUS THEORETICAL AND EMPIRICAL EQUATIONS

Antigen added	Antigen pptd.	Antibody pptd. (observed)	"Free antibody" (p)	Antibody pptd. (eq. 1)	Antibody pptd. (eq. 2)	Antibody pptd. (eq. 3)	Antibody pptd. (eq. 4)	Antibody/antigen in ppt. (R)
0.05	all	0.066	0.557	0.064	0.068	0.066	0.066	1.32
0.10	all	0.128	0.495	0.125	0.127	0.128	0.127	1.28
0.15	all	0.175	0.448	0.182	0.180	0.189	0.184	1.16
0.20	all	0.229	0.394	0.236	0.229	0.244	0.236	1.14
0.30	all	0.324	0.299	0.333	0.318	0.324	0.324	1.08
0.50	0.338	0.362	0.261	0.367	0.349	0.375	0.352	1.06
0.70	0.616	0.506	0.117	0.545	0.505	0.505	0.498	0.82
1.00	0.946	0.575	0.048	0.614	0.605	0.464	0.576	0.61
1.20	0.998	0.580	0.043	0.611	0.612	0.449	0.579	0.58
Σx^2	—	—	—	4.428	2.156	29.991	0.297	—

Amounts are given in milligrams.

The equations used for the calculations of the predicted values of antibody precipitated were:

(1) $R = 1.325 - 0.714\ G_a$ (80, 81)

(2) $R = 1.58 - 0.968\ G_a^{1/2}$ (80).

(3) $R = 14.84\ p/(1 + 957\ p)$ (65, 66, 124).

(4) $R = 1.57\ p^{0.31}$ (63).

In these equations R = the ratio by weight of antibody to antigen in the precipitate, G_a = antigen added, p = the concentration of unprecipitated antibody (mg. in each 2 cc.), shown in column 4 (obtained by subtracting the values in column 3 from 0.623), x = (true value of antigen precipitated − predicted value) × (10^2).

The expected values of "antibody precipitated" are calculated from the predicted values of R by multiplying by the amount of antigen precipitated.

[a] Data from (156).

where G means antigen precipitated, and other symbols have the same significance as in Table XXXVII. This will fit data in only part of the zone of antigen excess, before the excess becomes so large that the amount of antigen precipitated begins to fall off.

Hershey (91–96) has derived equations from slightly different considerations which also fit observed data fairly well, and Pauling *et al.* (154) have proposed an even simpler derivation.

More recently Teorell (173, 174) has predicted antibody–antigen relationships, assuming antibody to be univalent, and obtained very good agreement with various sets of data over the entire range (Fig. 40). It thus appears that we can hardly consider that the success of an equation in this field does much to support the theoretical bases on which the equation is based. The fact seems to be that data are too few, and subject to too many errors, for theories of serological reactions to be tested in this way. Serology is still a long way from being an exact quantitative science like physics.

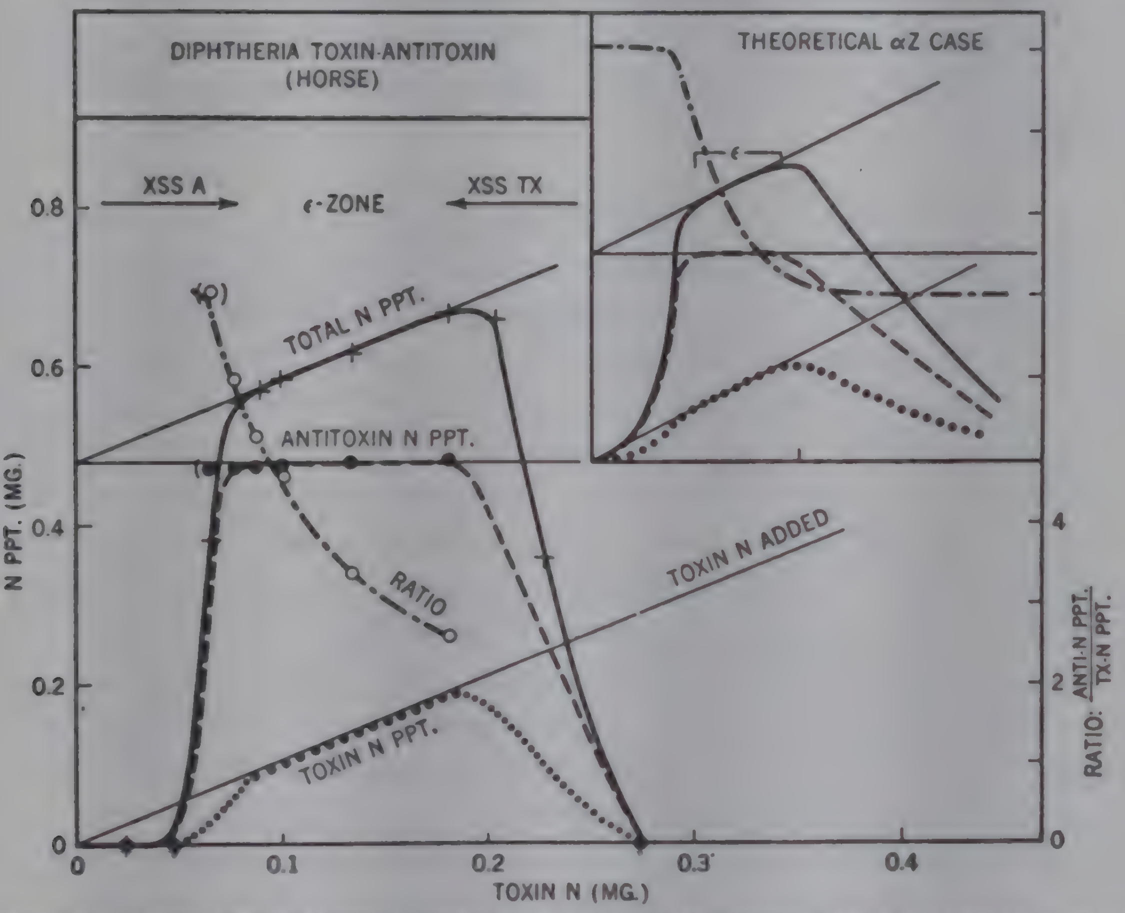


Fig. 40. Results of attempting to fit a theoretical curve (173, 174) to the *whole* range of the precipitin reaction.

Absolute proof that the alternation mechanism operates must presumably await further experimental evidence; in particular analytical proof that the valence of antibody is higher than one is practically essential. There are a number of experiments which seem on the whole more favorable to the alternation than to the Bordet theory. We have already discussed the observations of predominantly homogeneous aggregates in some mixed agglutination experiments, and failure of agglutination in mixed systems to be accelerated. We have also mentioned observations indicating that the second stage of many serological reactions seems to be specific.

Another experiment that favors the alternation hypothesis is Heidelberger's (74) observation that type 1 pneumococci, agglutinated with an excess of antibody and washed until detectable free antibody does not appear in the washings, then resuspended, are reagglutinated by the addition of fresh type 1 pneumococci, or type-1-specific polysaccharide. Such reagglutination did not follow the addition of heterologous type 2 pneumococci or polysaccharide. This was taken to show the role of intermediate links of antigen in building up aggregates.

SUMMARY

The reaction between antibody and antigen is a chemical one. (*1*) The basic facts about the reaction are: (*a*) the reaction is specific, (*b*) the entire molecules are involved, (*c*) the antibody and antigen molecules seem to behave as fairly rigid ellipsoids, (*d*) no splitting, digestion, or other profound chemical change takes place in either antibody or antigen, (*e*) the union is effected at the surface of the molecules, (*f*) the union is firm, but at least partly reversible, (*g*) both antibody and antigen enter into the final product, (*h*) antibody and antigen can combine in various proportions, and (*i*) there are certain analogies with colloidal reactions. (*2*) The reaction is considered to occur in two stages, combination between antibody and antigen, followed by an observable reaction (if any such occurs) such as precipitation, agglutination, complement fixation. There are several reasons for retaining this division into two stages: (*a*) the first stage proceeds without visible alteration of the mixture, (*b*) in the case of many small haptens no second stage follows the first, (*c*) under some conditions, such as in the absence of electrolyte, the first can take place but the second can not, (*d*) the speeds of the two stages are very unequal, the first being extremely rapid and the second often slow, (*e*) the energy change seems to take place mostly during the first stage, (*f*) the specificity of the second stage sometimes seems to be of a lower order than that of the first. (*A*) *First Stage: Combination.* (*1*) The forces holding antibody and antigen together are probably largely polar forces (coulomb forces between positive and negative charges), but other forces, such as the attraction between dipoles and dipoles, dipoles and ions, and the hydrogen bond, may play a role. (*2*) As in colloidal reactions, the reagents can combine in varying proportions, and the addition of an excess of one reagent gives compounds richer in this reagent, but not (outside of a narrow range) richer in proportion to the excess added. Electrolytes are important for both sorts of reactions, the reactions are often slowly or imperfectly reversible, and precipitation, optimal for certain proportions of the reagent, often occurs. (*3*) The size of the reacting groups in natural proteins is not exactly known, but we get some idea from the study of artificial conjugated antigens, and may hazard a guess that they are usually about the size of ordinary synthetic organic compounds, or sometimes a bit larger. (*4*) The reactive group is probably chemically complex, and the groups of the antigen and the antibody probably correspond, as a mold to a cast, in an electrical way, and perhaps spatially also. (*5*) Serological reactions may often be partially reversed by changes in electrolyte concentration, in temperature, etc. The difficulty of doing so varies with the system under examination. It is not likely that the union becomes appreciably firmer with the passage of time. (*6*) Combination is virtually complete after the lapse of a few minutes, perhaps a few seconds. The velocity of combination perhaps falls off exponentially with time. (*7*) The heat of serological reactions is rapidly evolved, and is large per mole of reagent, as would be expected from our ideas of the reactive groups and the nature of the union. (*8*) Various factors may affect the first stage, but it is usually difficult to distinguish this from effects on the second stage. Effects of *p*H, salt concentration, and temperature have been studied. (*B*) *Second Stage: Terminal Phenomena.* The second stage includes precipitation, etc. (*1*) Complement fixation is discussed in Chapter VII. (*2*) Lysis can be observed with bacteria, but better with erythrocytes. Hemolysis ("laking") is release of hemoglobin from the inside of the red cell. The stromata remain undissolved, although altered in shape, size, and osmotic properties. Lysis with antibody requires complement also; lysis can be produced also by nonspecific organic and inorganic substances not studied here. The amount of antibody required to lyse a cell is small, perhaps only about 30 molecules. (*3*) When the term bacteriolysis is used the killing of the microorganisms is generally meant. Not all bacteria are killed by antibody plus complement, but the cholera vibrio, the typhoid bacillus, and most Gram-negative bacilli are susceptible. A "prezone" may be observed (failure of higher concentrations of antibody to kill). (*4*) Antitoxins neutralize toxins, combining with them in varying proportions determined by the amounts mixed. It is not known if it does this by combining with the toxic groups *per se.* The toxin is not destroyed, for it can be recovered. The quantitative course of toxin–antitoxin reactions is at least superficially similar to that of adsorption reactions. Therefore the quantitative results are not simple like those of neutralizing acid with alkali, but the amounts of antitoxin required to bring about a certain fraction of complete neutralization are successively greater as neutralization is approached. The Danysz phenomenon consists in the fact that the toxicity of the final mixture depends on the way in which the reagents are mixed. If toxin is added to an otherwise adequate amount of antitoxin in portions, the mixture may be toxic, suggesting that most of the antitoxin was expended in neutralizing the first portions. This and the previous observation are probably the results of the power of toxin to combine with antitoxin in multiple proportions. The curative effect *in vivo* of an antitoxin may be greater or less than that calculated. Kraus suggested that, in addition to the amount of antitoxin in a serum, another characteristic, the *avidity,* might determine rate of neutralization. The important thing may however be the firmness of union with the toxin, or the two powers may be related. Less potent "low-grade" antibody has also been found in other instances. (*5*) An agglutinating antibody combines with cells and the cells stick together (agglutination). Bordet supposed the combination sensitized the cells to agglutination by the electrolytes present; the newer alternation hypothesis supposes that agglutination comes about as the result of the building up of a sort of "framework" of multivalent cells and multivalent (or divalent) antibody (see Appendix). Relatively little antibody is sufficient to agglutinate cells, but, if it is available, more will be taken up, up to a substantial fraction of the amount of cells used. If an agglutinating serum is diluted enough, it no longer agglutinates (postzone); some antisera show a "prezone" if the serum is too concentrated. The prezone has been found in some cases to be due to something which forms a thin film over the antibody–cell complex, preventing them from adhering. Changes in the conditions may affect agglutination, (*a*) salts are necessary for agglutination, but too much hinders, so a salt "optimum" is obtained, (*b*) an optimal *p*H range for agglutination, which is usually lower when the antibody concentration is low, (*c*) temperature increases the rate of agglutination up to about 30°C. or more, (*d*) shaking and stirring accelerate agglutination, (*e*) nonspecific serum proteins have little effect. (*6*) Precipitates often result when soluble antigens are mixed with their antibodies. The amount of precipitate depends upon the absolute concentration of the reagents and the ratio in which they are mixed. As in agglutination, zones are observed. Horse antiprotein sera exhibit a prezone (no precipitate if too much antibody is added). Rabbit antisera do not generally do this. If antigen is in excess, all systems exhibit a "postzone" which was formerly called a "prezone," but which is better known as the "inhibition zone" (Heidelberger). The equivalence zone is defined as the range of proportions where neither antibody nor antigen (or minimal traces of both) is to be found in the supernatant. The whole range of the precipitin reaction is thus divided into several zones (page 235). In the case of the majority of antigens, the great bulk of the precipitate consists of antibody; with antigens of high molecular weight antigen may predominate. A theoretical relation between the ratio of antibody to antigen (at the midpoint of the equivalence zone) and the molecular weight of the antigen has been proposed; it fits the known data fairly well. When the proportions in which the two reagents are mixed are varied, precipitates of varied composition result. If the amount of one reagent is kept constant and the other varied, data which can be fitted by "adsorption" or other similar equations are obtained. In order to study the composition of precipitates made in the region of antigen excess, it is best to use an antigen which contains no nitrogen, or else has an inorganic "marker" such as copper or arsenic. The molecular composition of analyzed precipitates can be calculated if molecular weights are known (page 243). Specific precipitates have a slight solubility which seems to decrease with the number of washings. Alteration in the conditions of precipitation affects the amount and composition of the precipitate: (*a*) precipitation does not seem to take place in the absence of salt, but stronger salt concentrations reduce the proportion of antibody—this knowledge may be utilized in dissociating antibody from specific precipitates formed at ordinary electrolyte concentrations; (*b*) precipitation can occur in the *p*H range 4.5–9.5, and the amount of precipitate seems hardly affected by *p*H within the range 6.6–8.0; (*c*) temperature generally has little effect in the range from 10° to 40°C., but in some systems the precipitate is partly or completely soluble at temperatures of 37°C. or over; (*d*) lipides have been reported necessary for precipitation in certain systems. Study of the *velocity of precipitation* has practical importance, as in the Ramon titration of toxins and antitoxins. In this titration antigen is kept constant and antibody is varied (β procedure). Dean and Webb introduced the α procedure, in which antibody is constant. An optimum (ratio of reagent concentrations giving most rapid precipitation) may sometimes be found by either method. Experience, and theoretical arguments, show that the α and β optimum can never actually coincide, but may not differ much in systems such as (horse) antitoxin–diphtheria toxin. It has been proposed to distinguish two classes of antibodies (R and H), depending on whether the system gives only the α optimum, or both the α and β. The equivalence (or neutral) zone may be located at or near one of the optima, but in some systems the zone is quite different. There has been observed, in the zone of antibody excess, an inverse linear relation between the antigen concentration and the time of flocculation. Changes in the conditions of precipitation may affect the velocity: (*a*) there is always an optimal electrolyte concentration, somewhat different for different electrolytes, (*b*) there is usually an optimum *p*H, (c) increase in temperature rapidly increases the rate of flocculation, up to 30°C. or more. Temperatures of 56° or higher may slow flocculation, perhaps by denaturation of one or both of the reagents, (*d*) shaking and stirring markedly increase the velocity of flocculation, (*e*) lipides seem to be necessary for flocculation, (*f*) nonspecific serum proteins have little more effect than might be expected from the increased viscosity, (*g*) other substances in the serum may play some role. The mechanism of the precipitin reaction has been treated theoretically (see Appendix). Flocculation tests for syphilis furnish an example of practical application of the precipitin reaction to the diagnosis of disease. The reaction is due to the combination of a "reagin" in the blood of syphilitic patients with a lipoid "antigen" usually extracted from normal beef heart. The relationship of these to the spirochete is still uncertain. Certain substances, such as cholesterol, promote the reaction. (*C*) *Appendix: Hypothetical Mechanisms of Serological Reactions.* (*1*) Agglutination of bacteria is often easy to bring about by various means, so slight alterations in their surface properties might suffice. The effects of combination of antibody with the cells were supposed by Bordet to sensitize them to agglutination by the electrolytes present, and to lower the surface charge. The newer "alternation" hypothesis supposes that the cells are held together by links of divalent (or multivalent) antibody molecules. Observation and motion picture photography of agglutination suggest that both mechanisms may be involved at times. (*2*) There have also been attempts to explain the precipitin reaction on the basis of the nonspecific Bordet hypothesis, and the "alternation" (framework) hypothesis. The latter supposes that the process of aggregation is specific throughout. A number of experiments seem to favor the alternation hypothesis. *Mathematical arguments* have been proposed by various workers, including Arrhenius, Boyd, Ghosh, Heidelberger and Kendall, Hershey, Pauling, and Teorell. All have obtained equations which fit certain quantitative data, but empirical and "adsorption" equations often fit equally well. The available data are probably not numerous or accurate enough to enable hypotheses as to mechanism to be disproved by such quantitative comparisons of observed data with the results calculated from theory.

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Chapter VI

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CHAPTER VII

COMPLEMENT AND COMPLEMENT FIXATION

1. Complement

As has been mentioned in previous chapters, for the bactericidal and hemolytic reactions of immune sera, as well as for other cytotoxic effects, there is needed, in addition to antibody, a naturally occurring substance or group of substances from the serum, called variously complement or alexin. The former term is in somewhat more common use in English. To give a specific example, if the complement of a serum hemolytic for sheep erythrocytes is removed or destroyed, it will be found that the serum no longer produces lysis when added to sheep cells. Antibody is still present and active, however, as is shown by the fact that such a serum will usually still agglutinate the cells, or by absorption experiments it can be shown that the antibody present is still capable of combining with the cells. If fresh normal serum, often even of a different species, is added to the mixture of serum and cells, lysis may be obtained, of a degree indicating that destruction of the complement did not decrease the amount of antibody present. Lysis will also be observed if the cells are removed from the "inactivated" serum and the fresh normal serum is added to them, without any uncombined antibody's being present.

Ehrlich believed that in lysis the antibody acted only as a go-between for the complement, which he believed to be the cause of lysis. He conceived the antibody as having two combining groups, one for the cell and one for the complement, and therefore called it an "amboceptor." Bordet denied that the antibody thus acted as a link, and considered that the complement united (by adsorption) because of some physical change in the sensitized cell.

Heidelberger, Weil, and Treffers (42) believe that the complement components possess one or more groupings capable of forming loose dissociable unions with individual antibody (and perhaps antigen) molecules, but that these unions become stable enough to detect only when the complement molecules are more or less surrounded by antibody (and perhaps antigen) molecules.

That the combination of complement with the sensitized cell may in part be due to nonspecific physicochemical forces is indicated by observations that complement will lyse red cells after they have been treated with colloidal silicic acid (2, 52), tannin (61), etc.

2. Mechanism of Action

The mechanism by which complement produces its effects is not known. It was supposed by Ehrlich that some sort of digestion followed its combination with the sensitized cell. Bordet (1) stated that the stromata of lysed erythrocytes no longer act as osmotic membranes. But complement does not appear to be identical with serum protease (48), and attempts to demonstrate any digestive action have been unsuccessful. There is no evidence that complement exerts any lipolytic activity.

Haurowitz and Yenson (36) compute that about 6000 molecules of complement are required to hemolyze a single erythrocyte. This indicates that complement is really a very efficient hemolytic agent, for more than 10^{10} molecules of saponin or oleate are required.

3. Species Differences

Since normal serum can be used as a source of complement, it might be imagined that its action is entirely nonspecific, and it is a fact that complement from the same serum can often be used to activate a variety of reactions involving a number of different antibodies and antigens. Nevertheless, complements are not all alike, and more or less well-marked species differences can be observed, a fact already known to Bordet and Ehrlich.

Muir (53) studied the power of activation possessed by various complements with antisera from the rabbit, cat, goat, duck, and ox. The antigenic cells in each case were the erythrocytes of the ox; the antibody in the ox serum was thus an isolysin (page 31). All of the antisera, except the duck, were activated by all the complements except those of the ox, pigeon, and horse. The horse complement was effective with cat serum. Muir considered that failure of sensitized corpuscles to lyse with a given complement might be due either to failure of the complement to combine, or insensitivity of the corpuscles to its action. Thus horse complement failed to give lysis with ox cells sensitized with rabbit antiox serum, but it could be shown that nevertheless a considerable amount of the horse complement had combined.

Dingle, Fothergill, and Chandler (12) in studying the bactericidal action of antisera of the horse, rabbit, and guinea pig against *Hemophilus influenzae*, found that in the case of horse antiserum, of the complements tried (guinea pig, human, and rabbit), only the human complement would activate the killing power; in the case of rabbit antiserum both rabbit and human complements activated, while, with guinea pig antiserum, guinea pig complement was also effective, although not more effective than the human complement. Most striking of all, horse complement did not activate horse antiserum.

Some individual differences in complement content of the blood have been found. Apparently these are sometimes hereditary, for a complement-deficient strain of guinea pigs has been investigated (46). It was found that this inactive guinea pig serum could be activated with fresh human and guinea pig sera, and to a less extent with dog, rabbit, and cat sera, but not at all with the sera from a large number of other species. It could be activated by heated human serum about as readily as by fresh, but five times as much heated normal guinea pig serum was needed, and other heated sera had no activating effect. The observation of Goodner and Horsfall (30) that antibodies for pneumococcus polysaccharide could be divided into classes, according to whether or not they fixed guinea pig complement, has been referred to in Chapter II.

Even the complement from a given individual of a given species may vary in its efficacy when tested with different systems. In general one of the best sources of complement for hemolytic systems is guinea pig serum, but we have just seen that this was found to be inefficient in the *H. influenzae* system. It is also a poor activator for the lysis of guinea pig corpuscles by anti-guinea-pig serum. It is in fact often found that an animal's complement is weak in activating the lysis of the blood cells of its own species, although this cannot be stated as a general rule. The relationships of the complements of different species have recently been discussed by Cushing (7, 8). See also page 291.

4. ORIGIN

It was early suggested that complement might be formed in the leucocytes, but attempts to extract it from leucocytes failed, and present day evidence indicates that complement is composed of normal constituents of circulating blood plasma. Since it is apparently a mixture of globulins and a mucoprotein (see page 291), it may be supposed that its origin is in the main the same as that of other globulins. Possible participation of the reticulo-endothelial system in the formation of complement is suggested by experiments of Jungeblut and Berlot (49), who found a fall in the complement content of the blood following "blockade" with india ink. However, the level came back to normal in 24 hours. Work such as that of Ehrlich and Morgenroth (26) and Dick (11) with poisons affecting the liver suggests that this organ takes part in the formation of complement.

5. Nature of Complement

The lability of complement early suggested that it might be protein in nature. It has been found (20) that as much as 90% of the complement activity of fresh guinea pig serum can be separated in a fraction which proves to be a constituent of the serum globulins. Contrary to the opinion of some earlier workers, Ecker *et al.* (20) do not believe that any of the serum albumins are intimately associated with the complement function. The fraction called "albumin" by other workers is said by Ecker and Pillemer (19) to consist of a mucoeuglobulin. These experimenters were able to separate complement into four fractions, of which two could be well characterized as proteins. Their properties will be discussed below.

It is still uncertain whether other serum constituents, such as lipides, constitute part of the substances we refer to as complement, but this possibility does not seem likely at present.

6. Effect of Various Reagents

One of the most striking things about complement is that heating to 56°C. for half an hour destroys its activity, although most of the serum proteins resist this treatment. The inactivation may not be entirely irreversible, for Gramenitzki (32) found a gradual return to an active condition after moderate heating.

Complement is also inactivated readily by various other treatments, such as prolonged shaking and addition of various salts. The activity is permanently destroyed by the addition of any considerable amount of acid or alkali, and in fact complement seems to have maximal stability only within the *p*H ranges 6.0 to 6.5 (6). It has also been observed to be destroyed by ultraviolet rays and α-particles. Other more or less destructive agents are proteolytic ferments, cobra venom, shaking with ether or chloroform, alcohol, alcoholic tissue extracts, bile salts, soaps, and some alkaloids. Complement activity also disappears if the serum is simply allowed to stand. Under ordinary conditions of icebox storage, as much as 90% of the activity may disappear in three to four days.

Complementary activity may be removed by absorption with many substances in the particulate or colloidal state, such as casein, cholesterol, kaolin, shellac, kieselguhr; inactivation also follows filtration through a Berkefeld filter (references in 3).

7. "SPLITTING" OF COMPLEMENT

It has been found (references in 63) that, as electrolytes are removed from serum, complementary activity is lost. Under these conditions the euglobulins are precipitated, and it is logical to suppose the complement has gone into the precipitate. However, if the precipitate is redissolved in saline, it alone does not have complement activity. But, if the dissolved precipitate is added to the supernatant from which it was precipitated, the mixture is active, although either component alone was inactive. It was found that the fraction precipitated would unite with sensitized red cells, although it did not cause hemolysis, while the fraction remaining in solution would not unite directly with the sensitized cells. Therefore the precipitated fraction was designated as "midpiece" and the soluble fraction as "endpiece." Heidelberger (40) in agreement with Pillemer and Ecker (55) designates these as C′1 and C′2, respectively.* The midpiece is adsorbed by lead phosphate and by titanium dioxide (19). Kaolin and magnesium hydroxide adsorb both midpiece and endpiece. It was also shown that it is the midpiece which is chiefly absorbed when antibody–antigen reactions take place, most of the endpiece remaining free. Both of these fractions are destroyed by heat. Saturation with carbon dioxide has also been used to separate these two fractions.

One of the agents which will absorb complement activity from serum is yeast cells. Zymin (dried, powdered, ether–acetone extracted yeast) will do the same thing. It was found by Coca (5) that a serum so treated could be rendered active again by adding a serum which had been inactivated by heating to 56°C., which we have just seen destroys both endpiece and midpiece. Thus a relatively heat-stable fraction of complement appeared to be demonstrated (C′3). It seems to be part of the midpiece (18). According to Ecker and Pillemer (19), it is destroyed by heating to 63° for 30 minutes. The hereditary complement deficiency of certain guinea pigs (see page 287) is apparently due to lack of this fraction.

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* The symbol C′, instead of C, is used because the latter symbol is used for certain bacterial antigens.

Gordon, Whitehead, and Wormall (31) have reported the presence of a fourth complementary factor, also heat stable, but not absorbed by yeast (C′4). It is specifically inactivated by treating the serum with ammonia. This factor was found by Gordon *et al.* to be essential for lysis, but not for opsonic (see page 13) action. Ecker and Pillemer (19), however, found that removal of C′4 destroyed the opsonizing action.

Pillemer *et al.* (19, 56) were able to obtain C′1 (midpiece), C′2 (endpiece), and C′4 in pure form, as well-defined proteins, by fractional precipitation of guinea pig serum with ammonium sulfate and dialysis. C′1 proved to be a globulin, with a sedimentation constant of 6.4 and an isoelectric point of *p*H 5.2–5.4. It was destroyed by heating to 50°C. for 30 minutes. C′2 and C′4 were obtained together in the form of an apparently pure mucoeuglobulin, containing 10.3% carbohydrate, with an isoelectric point of *p*H 6.3–6.4. The C′2 activity was destroyed by heating to 50° for 30 minutes, the C′4 activity by heating to 66° for 30 minutes. C′3 was found in all the globulin fractions, even to some extent in the albumin. It seemed to be a phospholipide or phosphoprotein.

Of these fractions, C′1 was found to comprise 0.72% of the total serum proteins, and the mucoeuglobulin corresponding to C′2 and C′4 comprised 0.17% of the total serum proteins. The general properties of these preparations are shown in Table XXXVIII. Recent advances in the chemistry of complement have been discussed by Pillemer (54).

At a temperature of 1°C., components C′2, C′4 and C′1 combine with sensitized sheep erythrocytes, while C′3 does not combine. C′1 will combine with sensitized cells in the absence of C′4 but is hemolytically inactive unless C′4 combines previously or simultaneously. C′4 does not combine in the absence of C′1 (57).

Although C′3 is not fixed by sheep cell–antibody complexes, it is nevertheless essential for hemolysis, and operates on the sensitized cell after C′1, C′2, and C′4 have combined. C′3 seems to behave like a catalyst.

The chemistry of human complement has been studied by Pillemer, Ecker, and co-workers (13, 14, 22–25, 54, 59, 62). Under the proper conditions and with the proper concentrations, all of the corresponding components of human and guinea pig complement are mutually substitutive (23).

TABLE XXXVIII

PROPERTIES OF PURIFIED COMPONENTS OF COMPLEMENT (56)

Complement components present	Euglobulin (mid-piece)	Mucoeuglobulin (endpiece and fourth component)
Mobility in phosphate buffer, at *p*H 7.7, 0.2 ionic strength	2.9×10^{-5}	4.2×10^{-5}
Sedimentation constant	6.4	—
Protein nitrogen, per cent	16.3	14.2
Carbohydrate, per cent	2.7	10.3
Phosphorus, per cent	0.1	0.1
Optical rotation	−28.7	−192.5
Apparent isoelectric point	5.2–5.4	6.3–6.4
Fraction of total complement activity, per cent	100	85
Fraction of total serum protein, per cent	0.6	0.18
Heat stability of complement activity (destroyed in 30 min. at tabulated (temperature), °C.	50	50[a], 66[b]

[a] For endpiece activity.
[b] For fourth component activity.

8. PRESERVATION OF COMPLEMENT

Since complement is so unstable, methods of preservation acquire practical importance. Some methods which have proved successful consist in the addition of sodium chloride up to 10%, addition of boric acid, or drying in the lyophilizing apparatus (page 428).

9. COMPLEMENT FIXATION

The exact mechanism of complement combination with antibody–antigen complexes, and the cause of the avidity which such complexes exhibit for complement, is still unknown. The older views of Ehrlich, already referred to, have been abandoned. It is evident from certain experiments (page 294) that the combination depends partly upon the size of the particles available for it to combine with. It is altogether probable that the physical state of the surface of the particles is also important, as is suggested by the experiments with nonspecific lytic agents (page 221), and the observation (45), made with antipneumococcus type 1 sera, that complement fixation with *guinea pig* complement was obtained in the presence of the homologous polysaccharide with sera of the rabbit, guinea pig, rat, and sheep, while fixation was not observed with sera of horse, man, mouse, cat, dog, or goat. The difference is evidently not a matter of the molecular weight of the antibody, for some of the animals in each of the above classes produce large molecules of antipneumococcus antibody, while some produce normal sized molecules (50). Dependence on physical state is also suggested by the observations that suspensions of a number of kinds of particulate matter will absorb complement, and the work of Hambleton (33, 34) who found that tubercle bacilli, by the addition of calcium chloride and magnesium chloride, could be made to fix complement avidly in the absence of antibody.

Heidelberger, Weil, and Treffers (42) have proposed the hypothesis that complement molecules, through groupings capable of loose dissociable union with antibody molecules (and perhaps antigen molecules, too), combine with antibody–antigen complexes in a purely chemical way. The unions are supposed to become progressively firmer because the complement molecules are gradually surrounded by antibody (and antigen) molecules as larger aggregates of antibody and antigen molecules are formed. These authors suggest that stabilization of the originally loose complement bonds "might result either through the attraction of approaching ionized groupings of opposite sign, through hydrogen bonding, through spatial accommodation of large groupings on C′1 (combining component of complement) and A (antibody), or through the presence, on C′1, as on antigen and antibody, of more than one grouping capable of reacting with A molecules brought into apposition." They make the interesting suggestion that possibly such union could be demonstrated without the presence of an antibody–antigen compound, or even with normal globulin, if there were any way of bringing a sufficeint number of such molecules into suitable apposition and holding them there. The anticomplementary power of purified γ globulin (9) seems in accord with this suggestion.

The chief objection to this point of view, aside from its purely hypothetical aspect, would seem to be the observations of Goldsworthy, cited on page 294, indicating that the maximal complement-fixing power of a mixture of antibody and antigen does not appear at once, as would be expected from the hypothesis of Heidelberger, Weil, and Treffers, but seems to develop only after the aggregates have had time to reach a certain optimal size. Also, Heidelberger *et al.* (41)

observed that resuspended, finely divided specific precipitates took up the complement-combining component from subsequently added guinea pig serum almost as well as precipitates formed in the presence of previously added complement. These observations are not too easy to fit into their proposed picture, unless we may assume that complement molecules can migrate into the interstices of aggregates after they have grown to considerable size, and be fixed there. The theory of Heidelberger *et al.* (42) did not specify the specific role played in the hypothetical union by the various components of complement. Obviously the actual course of events in complement fixation must be fairly complicated. Pillemer *et al.* (56a) have studied the role of the various components in fixation.

Quantitatively, the combination between antibody–antigen compounds and complement follows the adsorption equations, but this of course throws no real light on the mechanism of combination. It serves to explain, however, the "Ehrlich phenomenon," which is this: if we define for the sake of argument one unit of complement as the amount which, when added to a given system, leaves a little complement free after the reaction is finished, we find that much more than two such units have to be added if it is desired to leave a whole unit free. This may be compared with what was said above about antibody–antigen reactions, in particular that between toxin and antitoxin (page 224). The combination of complement and antibody–antigen compound is generally a firm one, with little dissociation (3).

The power of antibody–antigen compounds to combine with complement evidently depends on a number of factors. It has been mentioned previously that the species origin of the antibody and complement may have an important influence. The size of the antibody–antigen complex seems also to be a determining factor. Most precipitin reactions involving rabbit antibody fix complement as they progress, but it is not necessary that a precipitate should form for complement to be fixed, for this may occur in zones where there is no visible precipitation. Goldsworthy (29), who investigated this question, found that, as aggregation proceeded in precipitating systems, the power of fixing complement rose to a maximum, then declined, and, if complement was not added until precipitation was complete, little fixation occurred. Evidence was obtained which suggested that the maximal power of fixation was possessed by particles large enough to cause opalescence, but not visible to the eye. Mixtures in which antigen was in excess, and in which the particles never grew to the opalescent stage, fixed poorly or not at all. Dean (10) had previously observed failure to fix when antigen was in excess. Goodner and Horsfall (30) found no fixation when an antiserum against aminobenzylglucoside–protein was mixed with the uncoupled glucoside, although with the conjugated protein fixation was obtained. That is, the (invisible) hapten–antibody reaction did not fix complement.

Goldsworthy suggests that complement fixation is a function of the size of the particles of a precipitate. On this basis it is quite understandable that mixtures in which antigen is in excess, or deficient, so that the size of the particles increases only gradually, reach their maximal fixing power more slowly. Thus it was found that the relation of the complement-fixing optimum to the precipitating optimum (point of most rapid precipitation) depended on the interval allowed to elapse after the antibody and antigen were added, before the additon of complement. If complement was added simultaneously with the other two reagents (the usual technic), the fixation optimum was found either at the precipitation optimum (with weak or medium strength sera) or in a tube having somewhat less antigen (with very strong sera). If, however, an interval (20 minutes to four hours) were allowed to elapse before the addition of complement, the fixation optimum shifted in the direction of increased antigen, and was to be found in a zone where the particles had not grown too large to fix before the complement was added.

Eagle (15, 16) has explained the sensitization of the Wassermann "antigen" by serologically unreactive sterols as also simply a matter of particle size. He supposes the sterol acts as a nucleus around which are deposited reactive lipides of the antigen, resulting in the formation of larger particles which, when coated with reagin, fix complement better than the smaller particles formed in the absence of sterol. This is illustrated in Figure 41.

It is difficult to test whether fixation is influenced by the proportion of antibody to antigen in the aggregates, as well as by their size. It has been observed that the proportion of antiserum to antigen in the mixture may be widely varied, sometimes as much as 1000-fold, without notably lessening the degree of fixation. Experiments are on record in which a fixation optimum was observed with a constant amount of antigen; however in such experiments the complement was usually added simultaneously with the other reagents, so that the deciding factor might still have been particle size. With horse antihemocyanin antibody, where the antigen molecule is large, Hooker and Boyd (44) observed fixation in mixtures containing so much antigen that neither precipitation nor clouding would occur. In some of these mixtures there was only about one antibody molecule for each seven molecules of antigen; it is not known how far secondary aggregation might have proceeded in such a mixture, surely not far.

Combination of complement with antibody–antigen mixtures is rapid at first, but becomes slower with the lapse of time. The velocity, were it not for the deterioration of complement as the mixtures stand, would not fall to zero within less than about eight hours at 37°C. (15). The actual time of incubation of complement allowed with the antibody–antigen mixtures (usually 60 minutes at 37°, or four hours in the ice box) is essentially a compromise between the rate of fixation and the rate of destruction of complement.

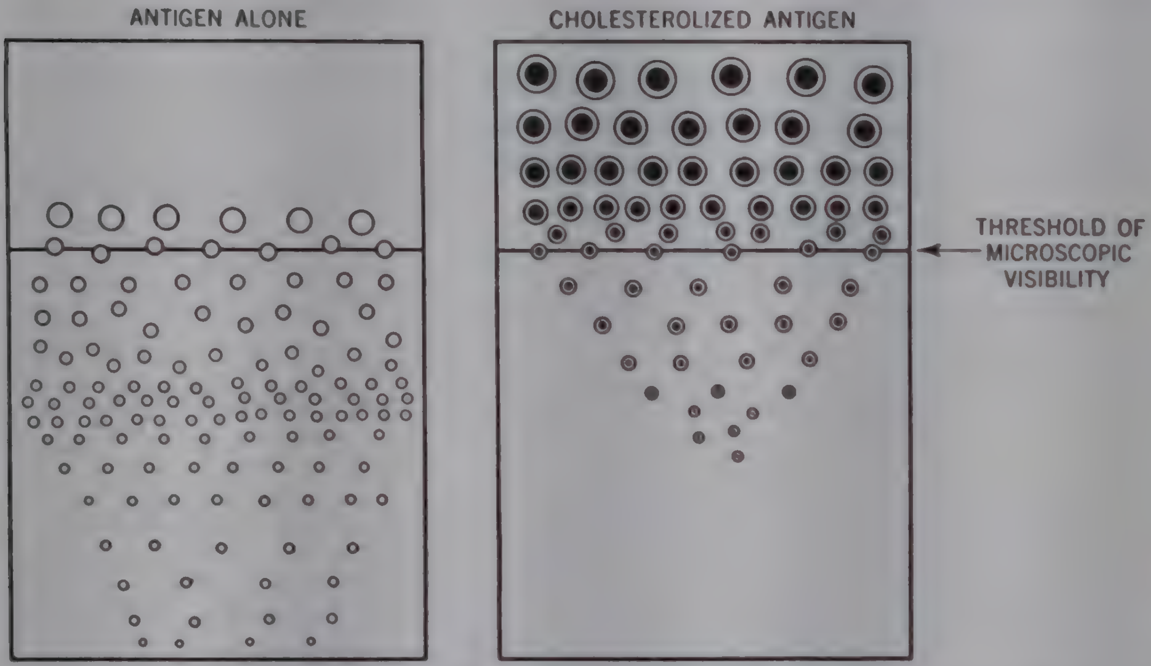

Fig. 41. The effect of cholesterol on size, number, and composition of particles in Wassermann antigen (diagrammatic) (15). The core (black) represents cholesterol or a cholesterol–antigen mixture, the clear area represents (lipoid) antigen.

The quantitative formulation of fixation reactions is complicated by the fact that the number of variables is large. It is possible to vary three different time intervals: the time elapsing after mixing the antibody and antigen before complement is added, the time of incubation with complement before the indicator (sensitized red cells–hemolytic system) is added, and the time of incubation following this addition before readings of the degree of lysis are made. (In practical work the first interval is usually zero, or negligible.) In addition, the amounts of five different reagents, the antibody, the antigen, the complement itself consisting of four components, the cells, and the sensitizer, may be varied.

The time intervals are however not usually varied much in work of this kind, so results of different experiments fortunately are comparable in this respect. The amounts of reagents are subject to more variation.

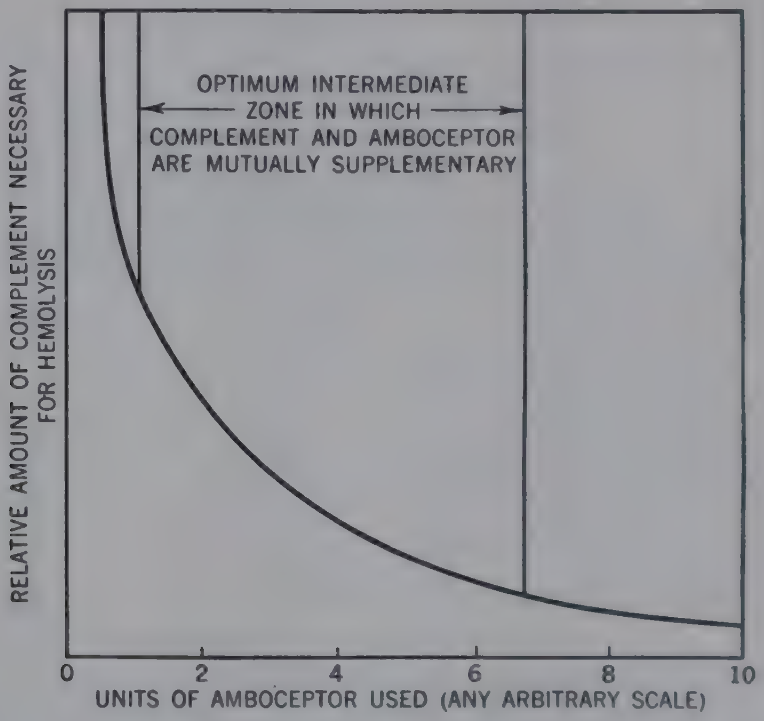

Fig. 42. Mutually supplementary activity of complement and lysin in hemolysis (diagrammatic) (31a).

A certain minimal concentration of antigen is requisite, but in the usual volumes fixation may be obtained with amounts of antigen of the order of 10^{-7} g., or less. A certain minimal amount of antibody must be combined with this antigen. More antibody than this does not usually prevent fixation, even in the case of horse antiprotein antibodies, where an inhibition zone with excess serum is observed in precipitation (44); but the fixation technic is such that large excesses of antiserum cannot be tried, as serum alone usually has some "anticomplementary" effect. With any given amount of (rabbit) antiserum, it is usually found that only amounts of antigen within a certain range will give fixation. With the lower concentrations of serum, this optimal zone contracts markedly, until with the smallest amounts it may be found that only one particular proportion of antibody to antigen will give complete fixation.

The more complement is used, naturally, the less complete the fixation is likely to be (i.e., more remains free in the mixture). Upon the amount of complement remaining depends the degree of hemolysis, if any, which is observed when the sensitized cells are added. However, the degree of hemolysis also depends upon the amount of sensitizer (hemolytic antibody) previously combined with these cells.

To a certain extent, complement and sensitizer (sometimes called amboceptor) can supplement each other in hemolysis. This is shown schematically in Figure 42, modified from Eagle (16). Of course, complete hemolysis cannot be obtained with less than a certain minimal amount of either; there is a certain optimal range of proportions for practical work, shown by the lines in Figure 42.

If too much sensitizer is used, hemolysis may be retarded (*Neisser-Wechsberg phenomenon*, or complement deviation). This is still unexplained. According to Ecker *et al.* (21), there is no relationship between the hemolytic complementary and the opsonic powers of serum.

10. ACTUAL WEIGHT OF COMPLEMENT INVOLVED IN FIXATION

Haurowitz (35) showed by determinations of the weights of precipitates made with and without complement that complement did not enter into the precipitate in anything like as large a ratio as one molecule of complement per one of antibody. Heidelberger (39, 40), using large excesses of complement, found that the presence of complement increased the amount of nitrogen in the precipitate by an amount corresponding roughly to one molecule per four molecules of antibody, if we assume complement and antibody to have the same molecular weight.

Pillemer *et al.* (58) found that the complement nitrogen which added to specific precipitates of pneumococcus polysaccharide S3 and its antibody came from components C′1, C′2, and C′4.

In terms of a "hemolytic unit," determined as the amount required for complete hemolysis of 0.2 cc. of a minimally sensitized 2–2.5% sheep red cell suspension, Heidelberger estimated that 1000 units corresponded to 0.10–0.14 mg. of complement nitrogen. If all the nitrogen comes from globulins, this would correspond to about 0.75 mg. of protein. It was concluded that guinea pig serum containing

200–250 units contains about 0.04 to 0.06 mg. of complement nitrogen per cubic centimeter, or about 0.4 to 0.7% of the total protein content of guinea pig serum. Some sera were found to have as much as 0.07 mg. nitrogen per cubic centimeter. Haurowitz and Yenson (36) computed that 1.5 × 10^{-14} g. of complement is required to hemolyze a single erythrocyte.

11. ANTICOMPLEMENTARY EFFECT

Most antisera, in sufficient amounts, and also some antigens, even without the addition of the other reagent, antigen or antiserum as the case may be, have the power of nonspecifically removing or inactivating some complement. This is called anticomplementary action, and often sets a limit to the amount of one or both reagents which can be used. The anticomplementary effect of the reagents used must always be carefully tested for by setting up proper controls.

There are probably a number of causes of anticomplementary action. It has been pointed out on page 292 that particulate matter often adsorbs complement. Sera which are old or contaminated with bacteria may be anticomplementary, and this property also may be induced by presence of soaps, acid, alkali, oxalate, etc. It has been found that absorption of antisera with bacteria (in operations designed to remove certain antibodies) may render them anticomplementary. Sera of syphilitic patients are likely to be intrinsically somewhat anticomplementary.

Hayes and Sachs (38) found that heating syphilitic sera tended to make them anticomplementary. They reported that addition of about eight volumes of .03 *N* hydrochloric acid precipitated out the anticomplementary fraction, leaving the syphilitic antibodies in solution.

The anticomplementary properties of human γ globulin, and the inhibition of this action by other substances, have been studied by Davis *et al.* (9). The anticomplementary action was decreased by heating to 56°C. for half an hour, and was abolished by the addition of approximately equal amounts of serum albumin or β globulin.

12. QUANTITATIVE FORMULATION OF COURSE OF THE FIXATION REACTION

Wadsworth, Maltaner, and Maltaner (64, 65) have studied the relation between the degree of hemolysis obtained and the amount of complement used. They report that, if to a given amount of antigen there is added an amount of antibody giving maximal fixation, a linear relationship is obtained between the concentration of antiserum and the concentration of complement required to give 50% hemolysis of the sensitized cells. Or, if a constant amount of antiserum is taken, and the amount of antigen taken which in each case gives maximal fixation, the relation is between the concentration of antigen and that of complement giving 50% hemolysis.

They found that the relation between concentration of complement (x) and percentage of hemolysis (y) could be represented by the formula mentioned by von Krogh (51), in which k and n are constants:

$$x = k\,[y/(1-y)]^{1/n}$$

This formula was found by von Krogh to fit very well data obtained by studying hemolysis produced with varying amounts of lysin, and various other serological and colloidal reactions.

Wadsworth, Maltaner, and Maltaner (66) have constructed tables based on the above formula, whereby from two readings of hemolysis the ratio c_{s+a}/c_s can be calculated, where c_{s+a} is the amount of complement required for 50% hemolysis with serum and antigen, and c_s is the amount of complement required for 50% hemolysis with serum alone. The numerical value of this ratio is taken as a direct measure of the fixing titer of the serum, and automatically allows for the anticomplementary activity. The higher the ratio, the more significant the degree of fixation observed.

13. Effect of Altered Conditions and Various Reagents

Temperature. Fixation is slow at 0°C., and increases rapidly with rise in temperature. The rate of deterioration of complement also increases very rapidly with higher temperatures, however, so that a limit is put to the temperature which can be used in practice. There are indications that a longer period of fixation at icebox temperatures gives a more sensitive reaction than incubation at 37°. According to Eagle (15) this depends on four main factors: (*a*) the time may be prolonged beyond that possible at 37°, (*b*) there is less inhibiting effect of the serum at low temperatures, (*c*) the anticomplementary action of serum is more pronounced in the prolonged low temperature incubation, favoring development of a positive reaction, (*d*) complement may deteriorate in the prolonged test, again favoring positive reactions.

It is apparent that these factors would be in favor of sensitivity, which may be desirable in a test for syphilis, for example, but might not necessarily make the test more reliable, which may be the important point in other instances where complement fixation is employed.

Time of Incubation. Fixation goes on rapidly at first, then more slowly, so that the amount of fixation increases with time. Here too, however, complement deterioration comes in as a limiting factor. Complement is destroyed with prolonged incubation, especially at room temperature and higher. Consequently, according to Eagle, the time allowed for fixation at 37°C. should not exceed 30 minutes. Longer times at lower temperatures favor the detection of weak positive reactions, as just mentioned, and are therefore favored by some for the Wassermann test.

Electrolytes. Complement is not fixed in the absence of electrolyte, and fixation is inhibited by excess electrolyte, e.g., over 1.5% sodium chloride. The optimal salt concentration, according to Eagle (15), is that commonly used in "physiological saline," viz., 0.85% sodium chloride. Some bivalent cations, even in traces, have a marked inhibiting effect (e.g., 0.03 *M* barium chloride or calcium chloride). Magnesium ions, however, may possibly be favorable in small amounts.

Hydrogen Ion Concentration. The optimal *p*H is substantially the *p*H of serum, falling in the narrow zone of 6.3–7.8. Complement itself is rapidly and irreversibly destroyed at *p*H values less than 5.5, or greater than 8.0.

14. FORENSIC COMPLEMENT FIXATION TESTS

We have previously said that the fixation of complement in a mixture can be used as an indication that an antibody–antigen reaction has gone on. The way this is used is shown schematically in Figure 43.

The value of this technic in cases where no visible reaction follows the combination of antibody and antigen is obvious, but the reaction has actually proved more sensitive than direct observation even in cases where visible precipitation could result. It has been found possible to detect by complement fixation amounts of a protein much too small to be demonstrated by direct precipitation with an antiserum. Therefore the method has found application in the determination of the species origin of foreign proteins, such as blood stains, in medicolegal cases. Anyone familiar with the technic of the Wassermann test, which is described in some detail in Chapter XI, can readily devise the appropriate modifications.

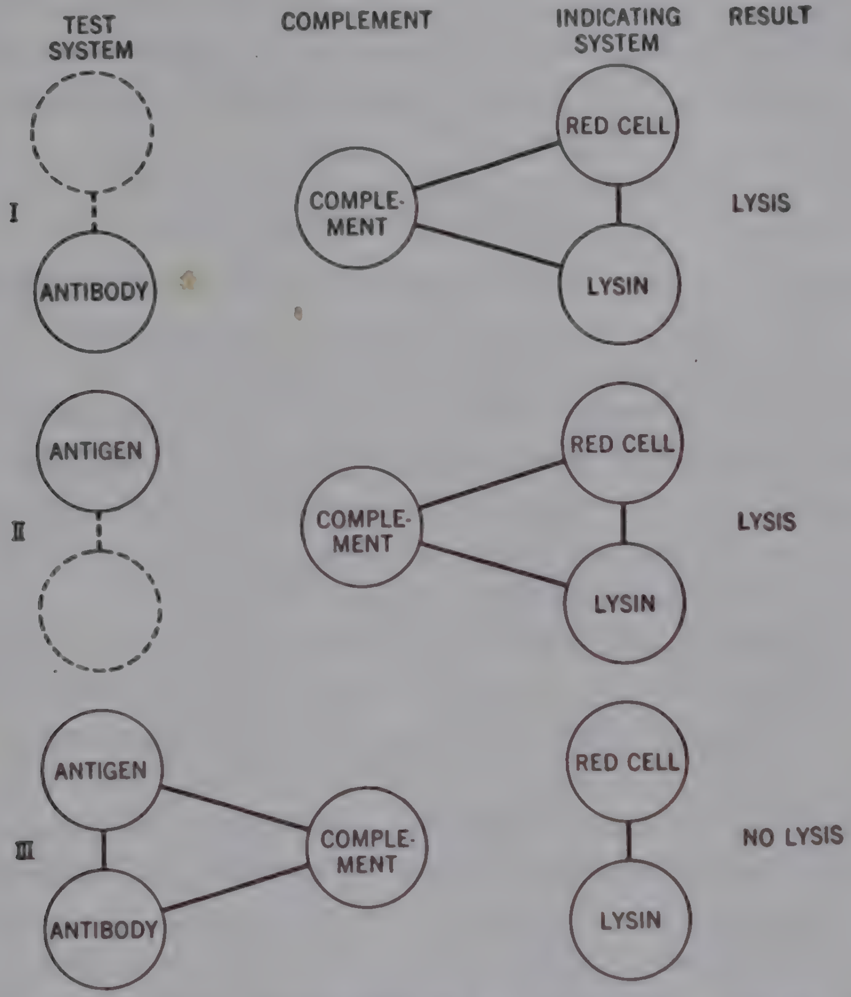
TEST SYSTEM
COMPLEMENT
INDICATING SYSTEM
RESULT
I
ANTIBODY
COMPLE-MENT
RED CELL
LYSIN
LYSIS
II
ANTIGEN
COMPLE-MENT
RED CELL
LYSIN
LYSIS
III
ANTIGEN
ANTIBODY
COMPLE-MENT
RED CELL
LYSIN
NO LYSIS

Fig. 43. Diagrammatic representation of complement fixation test: (I) test system contains antibody but no antigen, complement remains free and lyses sensitized cells added as indicating system; (II) test system contains antigen but no antibody, again complement is not fixed; (III) test system contains both antigen and antibody, complement is fixed, and sensitized cells added are not lysed. After Hooker (personal communication).

15. Applications to Diagnosis of Disease

Complement fixation has found valuable applications in diagnosis. The principle involved is the same as before. The question to be answered is generally, does the serum of the patient contain antibodies for the disease-producing agent that we suspect is causing his malady? By testing for complement fixation when the serum is mixed with the infectious agent or a suitable preparation from it, valuable information may be obtained. Of course since fixation is only a secondary reaction, consequent on the union of antibody and antigen, the fixation technic can not be expected to be more specific than other methods of detecting antibody–antigen reactions. Consequently cross reactions with related organisms, and (rarely) with antigens not closely related, may be found here as in other cases. The application of this technic to specific diseases will be mentioned in Chapter XI.

16. The Wassermann Test

Although developed before the flocculation tests for syphilis, the Wassermann reaction is best understood by referring to these reactions, which we have already discussed in Chapter VI. Wassermann *et al.* (67) used in their first work extracts of syphilitic organs as "antigen," but it was soon found that suitable "antigen" could be prepared from normal organs. The possible serological relation of the antigens now used, and of the reagin in syphilitic serum, to spirochetes, has already been mentioned in Chapter VI.

We may regard the Wassermann test as simply an application of the complement fixation technic to detection of the reaction between the lipoid "antigen" and the "reagin" in syphilitic serum. The reagents are mixed in the presence of complement, and, after suitable incubation, the indicating system, red cells sensitized by combination with hemolysin (amboceptor), is added. If hemolysis takes place, complement must still have been free when the sensitized cells were added, and no fixation had previously taken place during the incubation, or was not complete; if there is no hemolysis, fixation was complete, and we infer the presence of syphilitic reagin in the patient's serum.

In actual practice, the reaction must be carried out in a quantitative manner, with careful controls on all the reagents. The details will be presented in the chapter on technic, Chapter XI.

Mechanism of the Wassermann Reaction

Whether the reagin in syphilitic serum is a true antibody or not, we know that it combines with the lipoid "antigen" used. This can be demonstrated by adding an excess of the antigen to a Wassermann-positive serum and removing the lipoid particles (by high speed centrifugation or Berkefeld filtration). The serum will be found to have lost its positive reaction, or to have had it much reduced (15).

The lipoid particles after combination with reagin aggregate, as has been observed under the microscope by dark-field illumination (4, 47), even though no change visible to the eye alone is seen. The surface properties of the particles are found to be profoundly altered after combination with reagin. Their isoelectric point shifts towards that of serum protein, and their critical potential and the electrolyte concentration required to cause aggregation become the same as for particles of heat-coagulated protein. On the basis of these facts, Eagle (15) has suggested that the reagin forms a sensitizing layer of globulin around each particle. He believes that it is probably this sensitizing film which fixes the complement.

17. Applications to Other Diseases

The complement fixation technic can be applied to the diagnosis of various diseases. It is particularly valuable when the antibodies present refuse to manifest their activity in any other way (or only by neutralizing the infectious agent). Recent examples are furnished by the work of Enders (27) on mumps and of Witebsky *et al.* (68) on trichinosis. Fixation has also been used in influenza (28) and neurotropic viruses (37).

Summary

(*1*) Complement is a group of serum constituents needed for bactericidal and hemolytic effects, etc., of immune sera. Ehrlich thought complement lysed and antibody was just the link needed (amboceptor) between complement and cells. Bordet thought complement combined with sensitized cells only by adsorption. Combination of complement with cells treated with silicic acid, etc., supports Bordet; anticomplementary properties of γ globulin support Ehrlich. (*2*) The mechanism by which complement produces its effects is not known. (*3*) There are species differences in complements, although complement from one species may activate systems containing antibody from a number of different species. Serum of the same species may even fail to activate. Hereditary individual differences may be found. (*4*) The origin of complement may be supposed to be in the main the same as that of the other plasma globulins. (*5*) Lability of complement suggested it might be protein in nature; isolation of components confirmed this. (*6*) Various reagents will inactivate complement; heating to 56°C. for half an hour, prolonged shaking, acid, alkali, enzymes, adsorption, filtration, etc. Its activity disappears spontaneously when stored in the ice box (about 90% gone in three to four days). (*7*) Complement activity may be separated into components, C′1 (midpiece), C′2 (endpiece), C′3, and C′4. Purified components were found to be proteins. C′1 seems to be the main constituent which combines; C′3 does not combine but acts like a catalyst. (*8*) Complement may be preserved by use of sodium chloride, boric acid, or lyophilizing. (*9*) The exact mechanism of combination of complement with antibody–antigen complexes is still unknown. Particle size and species differences seem to be concerned. Heidelberger *et al.* have proposed a theory. Quantitatively, complement fixation follows "adsorption" equations. This makes the "Ehrlich phenomenon" clearer. The time which elapses is also a factor. To a certain extent, lysin (amboceptor) and complement may supplement each other in hemolysis. (*10*) Guinea pig sera contain about 0.04 mg. of complement nitrogen per cubic centimeter; about 1.5×10^{-14} g. of complement is required to hemolyze a single erythrocyte. (*11*) Some sera and antigens are anticomplementary (combine with complement without addition of rest of system). There are probably a number of causes of this, including the presence of particulate material, etc. (*12*) If carefully controlled amounts of all reagents are used, the quantitative course of complement fixation may be formulated. Comparisons of the amount of complement used by serum plus complement with that used by serum alone gives a quantitative expression of the degree of fixation. (*13*) Variations in the conditions under which complement fixation takes place affect the result; fixation is slow at low temperatures (but complement is less stable at higher temperatures); the rate of fixation is rapid at first but tapers off with time, electrolytes seem to be necessary; the optimal *p*H is about 6.3–7.8. (*14*) Complement fixation can be applied in forensic tests. (*15*) Complement fixation finds application in the diagnosis of disease (detection of antibody to a suspected organism). It may be more sensitive, but not more specific than any other antibody–antigen reaction, since it depends on this. (*16*) The Wassermann test is an application of complement fixation to detect the reaction between the lipoid syphilitic "antigen" and the "reagin" in the patient's serum. The reagin, whether or not a true antibody to the spirochete, combines with the "antigen" (which comes from normal animal tissues), and in doing so fixes complement. The absence of fixation is a negative reaction for syphilis. (*17*) Complement fixation can be applied to the diagnosis of other diseases, such as mumps, trichinosis, and influenza.

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Chapter VII

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CHAPTER VIII

ANAPHYLAXIS AND ALLERGY

The word immunology in the title of this book may have led some unwary readers to suppose that we were going to be interested only in mechanisms which increase the resistance of an animal to disease, or help protect him against the effect of deleterious foreign substances. This is not entirely true, however, for immunology includes the study of a number of conditions in which the animal is actually more, not less, susceptible. This is traditional and proper; the study of such states of heightened susceptibility belongs to our field, because they are conditioned in most, if not all, cases by mechanisms which show the closest similarity to those we have investigated above. For example, they exhibit the same high degree of specificity, can often be shown to depend on the presence of antibodies, and, like the ordinary sorts of immunity, often result from infection with a specific disease, or exposure to a specific foreign substance. It is not extending the meaning of the term too much to call these phenomena immunological.

A certain extension of the word immune has already been introduced, for we have spoken of a rabbit which has been repeatedly injected with egg albumin, for example, as immunized, although egg albumin is not a toxic substance for a normal rabbit, and although the "immunized" rabbit has not been particularly protected by the treatment. Indeed, as we shall soon see, an animal which has been injected with a harmless protein may actually become, not resistant, but highly susceptible to it, so that the later injection of a small dose may kill him with great rapidity. It may also be found that an animal which has been infected with a certain disease-producing agent, although apparently more resistant to a subsequent infection, may be definitely less resistant than the normal animal to injection of certain substances derived from the infectious agent, or to reinfection with massive doses of the living agent.

We shall also include a consideration of hypersusceptibility in man. Many of these conditions, as for example asthma and hay fever, have, so far as we can tell, no connection with infection. Patients suffering from these complaints find, to their great annoyance, that they have acquired an altered way of responding to certain constituents of their environment. These persons are more, not less, susceptible, yet their difficulties are produced by reactions which are essentially the same (in mechanism) as those which do come into play in defense against disease.

It may still seem wrong to some to define immunological processes as those concerned in increasing resistance to disease, and then claim the right to treat under this heading processes which may actually reduce the resistance of the individual, or cause him to have a disease which would otherwise not exist at all. This apparent paradox probably depends fundamentally on two facts: (*a*) In many of the cases where an individual suffers as the result of a newly acquired altered reactivity to some agent, the conditions are artificial. We may perhaps say poetically that Nature did not intend that human beings should have horse serum injected into their veins and tissues, and it need not cause surprise that the results of doing so are sometimes disconcerting as well as undesirable. The guinea pig is extremely easy to kill by an immunological reaction we call anaphylaxis, but it may be doubted if many guinea pigs under natural conditions ever died of anaphylactic shock. When we perform such an experiment we are setting up conditions which are essentially artificial; as Hill (43) has put it, we are "stacking the cards" against the guinea pig. (*b*) We must admit that many lines of evidence indicate that the immunological mechanisms are teleologically not perfect, and some of the disadvantageous effects of specifically altered reactivities are due to overfunction, or misdirected function, of one or another of these mechanisms. This is not a unique state of affairs; there are analogies for it in ordinary pathology. We may suppose that allergic disease, in cases where there can clearly be no question of advantage to the subject, is the result of the miscarriage of a process which under other circumstances, as in bacterial infection, may be of actual service to the organism.

There is good reason to believe that heredity in many cases markedly affects the type of serological reactivity of which an animal is capable, and in particular some types of human allergy are probably the result of definite inherited tendencies to become hypersensitive. So, when we speak of such conditions as the results of the misfunction of a normal mechanism, we should keep in mind the role of heredity in accentuating or possibly initiating this departure from the normal.

The phenomena described in preceding chapters, of great importance in the resistance of the host to disease, all had one characteristic in common, namely, that they could also be studied *in vitro*. The phenomena we are now to consider can be studied only *in vivo*, or at any rate only in connection with living tissue. It can easily be imagined how this increases the difficulties of experimental technic and interpretation.

Of course the chief reason for the study of the reactions of hypersensitivity is their clinical importance. However, even apart from this, it would be desirable to consider them, since we believe their mechanisms are fundamentally immunological in nature. Also, some of them, particularly anaphylaxis, provide a valuable experimental tool in the study of specificity and other serological problems.

A. Anaphylaxis

Portier and Richet (74) reported that toxic extracts of the tentacles of certain sea anemones, in doses so small that they produced no symptoms in normal animals, would, if injected into a dog which had recovered from a previous—sublethal—dose, cause violent illness and often death. To characterize the *lowering* of resistance thus evidenced, they coined the phrase, anaphylactic action (*action anaphylactique de certains venins*), thus calling attention to its antithesis to the prophylactic or protective effects following other forms of treatment.

Other workers (Magendie, Flexner, Richet, and Héricourt) had previously observed what was doubtless anaphylaxis; and Arthus, Theobald Smith, and Rosenau and Anderson subsequently noticed similar phenomena. It was discovered that the substance provoking anaphylaxis need not be toxic in itself; on the contrary, normal animal serum from a different species, or harmless substances such as egg white, would produce the effect. It was only necessary to "sensitize" the animal by one or more previous injections, and then, after an appropriate interval, administer the anaphylaxis-provoking, or "shocking," dose.

1. PRODUCTION OF ANAPHYLACTIC SENSITIVITY

Animals can be sensitized by most antigens. The majority of these, of course, are proteins, but Landsteiner and Jacobs (59) induced anaphylaxis by injection of arsphenamine (which has occasionally been observed to sensitize human beings), and Landsteiner and Chase (56) were able to sensitize animals anaphylactically by the cutaneous administration of simple compounds such as picryl chloride and 2,4-dinitrochlorbenzene. It had formerly been believed that haptens might shock, but would not sensitize.

Administering the sensitizing antigen (called the anaphylactogen) by any route which avoids its destruction by the digestive enzymes may be effective, just as in the case of antibody production. Sensitization has been obtained by administration of the antigen intravenously, subcutaneously, intracutaneously, and following absorption through the placenta, the skin, and the respiratory passages. Some sensitization is sometimes produced even by feeding the anaphylactogen, especially to scorbutic guinea pigs.

The amount of antigen required for sensitization varies widely. All that is necessary is enough to start antibody formation. Rosenau and Anderson (78, 79) found that 10^{-6} cc. of horse serum (about 7×10^{-8} g. protein) would sensitize a guinea pig, and Wells (103) got definite sensitization of small guinea pigs with 5×10^{-8} g. of crystallized egg albumin. These amounts appear to represent about the limits in this direction. Larger doses may be necessary to sensitize sufficiently for fatal shock; a single subcutaneous dose of 0.01 cc. of horse serum (about 0.001 g. protein) may be taken as a typical amount for practical work. Larger doses have been found successful, up to five to ten cubic centimeters of serum. If the dose is too large, however, especially if it is repeated, the animal may be protected, in some cases for weeks, from the effects of the shocking dose; this may perhaps be due to the persistence of antigen in the circulation so that the animal is in effect desensitized (see page 311). In animals such as rabbits, dogs, and cats, larger doses than those used for guinea pigs (one or two cubic centimeters of serum), repeated several times at two- or three-day intervals, are better, and in the rabbit the minute amounts found to be sufficient for guinea pigs are quite ineffective.

The time factor is important. With the standard dose of about 0.01 cc. of foreign serum, guinea pigs begin to be sensitive in about eight days, and the sensitization reaches a maximum in about three weeks. After this it declines, but remains sufficient to give fatal shock, provided a large enough shocking dose is used, for about six months. It is possible that the animal remains somewhat sensitive for life. In rabbits, which should be injected repeatedly, a ten-day to three-week interval should be allowed to elapse before attempting to elicit anaphylaxis; in dogs and mice the optimal interval is about three weeks.

2. Induction of Anaphylactic Shock

In addition to the method of sensitization, and the time allowed to elapse before test, the amount and mode of administration of the shocking dose are important. The most severe symptoms occur when the antigen comes suddenly in contact with the sensitive tissues. Therefore the best method of eliciting anaphylactic symptoms is by injection either intravenously or directly into the heart. In animals which are not particularly sensitive to anaphylactic reactions, such as rabbits and dogs, this is the only way in which death can be obtained with any regularity. In the guinea pig as little as 0.01 cc. of serum intravenously may produce fatal shock. Guinea pigs may be killed by injecting the shocking dose intra-abdominally or even subcutaneously, but considerably larger amounts are needed. The actual amount required will depend upon the degree of sensitization of the animal, and partly, therefore, upon the size of the sensitizing dose and the interval which has elapsed. If the degree of sensitivity is less, larger shocking doses are required. In all cases the shocking dose must be considerably larger than the minimal quantities which have sometimes been observed to sensitize.

It was once thought that shock could be produced only by protein antigens, but this is not always so; Landsteiner and van der Scheer (60, 61), followed by later workers (12, 70), were able to produce anaphylactic shock by injecting azodyes.

3. Desensitization

If the administration of the shocking dose does not result in death, after recovery the animal is temporarily refractory. It is no longer sensitive to similar injections of the antigen, and is said to be desensitized. In line with this, the onset of sensitivity in injected animals may be postponed by injections of moderate quantities of the antigen in the period just preceding development of hypersusceptibility. Administration of the antigen by a method allowing gradual penetration to the sensitized tissues will desensitize, as will the repeated administration of minute doses too small to produce any symptoms.

The desensitized state is only temporary. In guinea pigs it lasts two weeks or more, and in rabbits a much shorter time (83, 84), perhaps because of the greater rapidity of antibody production in the latter animal. Afterwards the animal becomes equally sensitive or more sensitive than before.

It is logical to suppose that the reason for the refractory state following specific desensitization is temporary saturation of tissue antibodies by antigen, so that when further antigen is administered nothing happens.

4. Nonspecific Desensitization

It has been found that anesthesia, or injection of large quantities of an unrelated antigen, or of one or more of a variety of substances (see list in 87), may depress the degree of anaphylaxis obtainable by injection of the shocking dose of the specific antigen. The desensitization is not absolute, for larger doses of the antigen will still shock. A number of workers have reported being able to desensitize with histamine (references in 50); Karady (50) was able thus to prevent anaphylactic death in many guinea pigs sensitized to horse serum but, remarkably, not in those sensitive to egg white. Doerr has suggested that nonspecific desensitization depends upon alterations in the condition of the smooth muscle. Others (110) have pointed out the similarity of such phenomena to the disappearance of cutaneous hypersensitivity to tuberculin and foreign proteins at the time of the rash in measles.

5. Specificity of Anaphylaxis

The specificity observed in this reaction is of the same order as that found in other serological reactions. After sensitization to an antigen, an animal reacts anaphylactically only to this antigen or to one chemically related. Animals may be made sensitive to a number of antigens at the same time, and will react separately to each of them. After desensitization with one antigen the animal still remains reactive to the others, consequently anaphylaxis is useful in experimental investigations of specificity. It was used extensively by Wells and others, notably in studies on the specificity of plant antigens. The *symptoms* of anaphylaxis, however, in a given species of animal, are not dependent on the antigen used.

6. Passive Anaphylaxis

It has been implied already that the sensitive condition in animals is due to the development of antibodies, and this is strongly confirmed by the observation that a normal animal can be rendered susceptible to anaphylactic shock by transferring to it serum from a sensitive animal. This serum need not necessarily come from another individual of the same species; in fact a very convenient experimental technic uses the guinea pig as the test animal, but sensitizes the animal with immune serum from rabbits. The guinea pig may usually be sensitized quite easily in this way (48), even though the rabbit which provided the serum might suffer no shock if the test dose of antigen were injected into it.

Under suitable conditions, it is possible to produce typical symptoms of anaphylaxis in guinea pigs by injecting the antigen, followed by the antiserum (see 54). This is called reversed passive anaphylaxis. In this case also an appropriate incubation period (4 to 24 hours) must be allowed to elapse, as in ordinary passive sensitization (see page 446). It has also been found that such animals can be specifically desensitized by subshocking injections of the antiserum.

Although it is clear that passive sensitization with immune serum is due to the transfer of some sort of antibody, the animal does not usually become sensitive immediately following the injection of serum from a sensitive or immune animal. It is generally found that before typical shock can regularly be obtained, an interval of a few hours must elapse, and maximal sensitization was not found by Kellaway and Cowell (53) before four to six days. Apparently the antibody must be allowed to "settle down" in the tissues before the animal becomes reactive. The failure of antibodies from certain species to sensitize the guinea pig might perhaps be due to their inability to attach themselves appropriately to the guinea pig tissues.

However Zinsser and Enders (109), studying the phenomenon of reversed passive anaphylaxis, made the observation that some guinea pigs could be shocked by antibody within a few minutes after the administration of the antigen. It was found that only certain stocks of guinea pigs exhibited this behavior, which thus appears to be hereditary. The same stock was also susceptible to shock in the ordinary way (antiserum followed by antigen) even when an interval of only a few minutes had elapsed between the injections. Rabbits have sometimes behaved similarly.

Dean, Williamson, and Taylor (27) also reported being able to obtain passive anaphylaxis by injection of antigen immediately after administration of the serum. The source of the guinea pigs used in these experiments was not mentioned.

Species differences are observed in passive sensitization experiments. Rabbit antisera will usually sensitize guinea pigs passively, but horse antisera have not usually been observed to do so (3, 8, 33), although Bailey (4) was able to sensitize with antipneumococcal serum. It has also been observed that guinea pigs cannot be sensitized with antiserum from fowl (34), and pigeons cannot be sensitized with antiserum from rabbits (36, 85, 99).

The optimal anaphylactic reaction in passive anaphylaxis is obtained (49) when the relation of antigen to antibody is such that, by the standards of the precipitin test, antigen is in large excess.

7. Symptoms of Anaphylactic Shock

The symptoms of shock vary in different species of animals. The rapidity of onset and severity of symptoms depend somewhat on the route of injection of the shocking dose. The animal may sometimes die in 30 minutes after intra-abdominal injection. Following intravenous or intracardial injection an even more rapid and violent reaction is obtained.

Guinea Pig. The symptoms of anaphylactic shock in the guinea pig have been extensively studied, and have been vividly described by Seegal (87): "After a pig has been injected intravenously with the . . . shocking dose of antigen, it shows signs of distress within a minute. The hair on the head and back of the neck begins to ruffle. The animal becomes restless, coughs and retches, rubs its nose, and seems to choke. Respirations, which were at first increased in frequency, become slower and labored and soon the animal is gasping for breath and making tremendous inspiratory efforts. The mucous membranes become cyanotic. The animal defecates and urinates. If the anaphylactic shock is destined to end fatally the animal soon becomes weak and rolls over on its side, gives a few convulsive kicks, gasps, and stops breathing. In very severe shock this symptomatic cycle may all be over within five minutes. In a less sensitive animal the symptoms of restlessness, peripheral irritation, and respiratory distress are followed by considerable weakness and a marked drop in temperature. The pig huddles in a corner with hair ruffled and attention centered on its respirations, which are still accomplished with difficulty. After fifteen to thirty minutes it begins to shiver and improvement followed by recovery gradually sets in. The onset of shivering is usually an indication that the body temperature is rising again. If instead of giving a sufficiently large injection of antigen to precipitate acute shock a minute amount is injected, of the order of 0.000001 cc. of serum, guinea pigs develop an elevation of temperature (Friedberger and Mita).

"When the shocking dose of antigen is given intraperitoneally the most marked symptom is generally weakness. The animal lies on its side or drags itself around feebly, while the respiratory difficulties are not so marked although never absent. Death may not occur for thirty minutes or more, and indeed animals may finally recover from very grave symptoms."

The blood pressure rises from the normal of 80 mm. to 90–140 mm., then gradually (in around ten minutes) falls to 10–20 mm. Asphyxia is the immediate cause of death in the guinea pig, and immediately after death the heart is still beating. The lungs are markedly inflated, due to constriction of the bronchial musculature. Small hemorrhages are common on the under side of the diaphragm and in the viscera. The coagulation time of the blood is found to be prolonged, and Zuntz and LaBarre (111) have reported hyperglycemia and increase in blood lactic acid during the first minutes of shock.

The Dog. The classic observations of Portier and Richet (74) were made using the dog as the test animal. Richet's (75) description is still one of the best: "In the mildest forms the only symptoms are pruritus, increase in the number of respirations, lowering of the arterial pressure, increased frequency in the movements of the heart, diarrhea, and tenesmus. . . . If anaphylaxis is profound . . . the first symptom is frequent vomiting, so prominent that in a number of cases it develops at the end of ten seconds. . . . This symptom is so characteristic that it may be taken as the criterion. The vomit is frothy and mixed with bile; sometimes it is fecal, and sometimes, in severest cases, mixed with blood. . . . There is fluid diarrhea mixed with blood. . . . Ataxia suddenly comes on; . . . (the dog) becomes paraplegic; drags the hinder part of its body. The pupils dilate and the eyes are dulled, and, after lamentable cries, the animal passes urine and feces, becomes exhausted and insensible. . . . Respiration is quickened and dyspnoeic; the arterial pressure is very low, scarcely 4 to 5 cm. of mercury. The heart hurries its beats, which are so weak that sometimes they can scarcely be counted. . . . The general condition is serious enough to believe death imminent, but in reality death in less than two hours is extremely rare in the dog."

It has been observed that in the dog the liver is profoundly congested during shock, and that in fact most of the symptoms observed

TABLE XXXIX

Pathology of Anaphylaxis in Different Species of Animals (87)[a]

Animal	Congestion and hemorrhage	Edema	Liver	Lungs	Right-sided heart failure
Guinea pig	Hemorrhages of stomach, cecum, lungs, heart, etc. (Gay and Southard).	Lung, skin (Schultz & Jordan, Ramsdell).	Occasional local fatty changes.	Emphysema due to constriction of bronchioles.	Questionable.
Dog	Liver, gall bladder, gastrointestinal tract, lungs, endocardium and pleura, auriculoventricular bundle (Dean *et al.*, Richet).	Intestinal mucosa (Manwaring, Beattie and McBride).	Congestion central necrosis (Weil).	Very occasional emphysema.	
Rabbit	Liver and gastrointestinal tract (Scott).	Slight of lung.	Marked engorgement of intralobular capillaries, central veins, and portal vein.	Very occasional emphysema. Proliferation and phagocytosis, endothelial cells (Domack).	Marked due to constriction of pulmonary arterioles (Drinker and Bronfenbrenner, Gilbert).
Rat	Gastrointestinal tract, lymph gland, etc. (Parker *et al.*).		Congestion	Very occasional emphysema.	
Mouse	Intestine and stomach (Ritz).			Moderate emphysema (Schultz and Jordan).	Present.

[a] Modified as suggested by Dr. B. C. Seegal.

are due to what happens in the liver. The intense congestion of the liver has been explained as due to injury of the liver sinusoids and liver cells, allowing transudation of fluid, edema, and congestion with red cells, and to constriction of the hepatic veins, producing interference with the outflow of blood.

The Rabbit. Death due to anaphylaxis is not so common in the rabbit. When it occurs it is usually rapid. The first sign appears to be a flush of the ears, followed by pronounced pallor. The animal lies with legs outstretched or falls on its side, gives a series of convulsive movements, often associated with the passage of urine and feces, and dies. The heart may continue to beat after respiration has ceased (87), or respiration may continue for a brief period after the heart has ceased to beat (96). Rabbits which have progressed to the stage of coma may sometimes recover. In nonfatal anaphylaxis the rabbit may show few symptoms other than an increase in respiration rate. However, the blood pressure and body temperature fall.

In the rabbit the pathological symptoms are different from those observed in either guinea pig or dog. The pulmonary dilatation found in the guinea pig is absent, and there are no marked hemorrhages in the splanchnic area, although some congestion of the liver and other viscera may be found. The most characteristic finding is the extreme dilatation of the right side of the heart and the inferior vena cava with blood, apparently due to constriction of the pulmonary arterioles (31, 37).

The Horse. The symptoms in the horse are dyspnea, rapid, feeble heart beat, and increased defecation and urination; and more important, urticaria, edema of the limbs, cyanosis, edema and petechial hemorrhages of the mucous membranes, lacrimation, and salivation.

Other Animals. Anaphylaxis in the mouse has been discussed by Weiser *et al.* (102). References to work on other animals will be found in (76, 87, 90).

Man. Fortunately there have been few cases of anaphylactic death reported in man. The symptoms of milder shock will be described in the following section on allergy.

8. Pathological Changes

A synopsis of the pathological changes following anaphylactic shock in various animals will be found in Table XXXIX, taken from an article by Seegal (87).

The pathological and physiological symptoms of anaphylaxis at first sight appear unrelated and surprisingly different in different species. However, it has been shown (87) that most of the observations can be referred to one or the other of two principal causes: contraction of smooth muscle and increased capillary permeability.

In the guinea pig and the rabbit the immediate cause of death is probably contraction of smooth muscle in the bronchi and in the pulmonary arterial system, respectively. In the dog, cat, guinea pig, rabbit, horse, rat, pigeon, and sheep, any one or all of the following symptoms: vomiting, diarrhea, or frequency of defecation, provide evidence of increased activity of the gastrointestinal tract. (Note, however, that rodents do not vomit.) The ruffling of the hair is presumably due to contraction of the *arrectores pilorum*.

Increase in capillary permeability may explain the hyperemia, hemorrhages, edema, and urticaria observed.

The fall in blood pressure may be explained partly by dilation of the capillaries and partly by smooth muscle contractions in various organs. In the guinea pig the blood pressure changes may be largely due to the anoxemia, following contraction of the bronchioles.

Cannon *et al.* (15) studying anaphylaxis in rabbits reported that the primary effect of the antigen–antibody reaction in the lungs was increased capillary permeability, followed later by severe vascular injury. In the liver Hartley and Lushbaugh (41) found massive areas of coagulative necrosis of the parenchyma.

The organs chiefly involved in anaphylaxis are called the "shock organs." The differences observed in various species may be due partly to differences in the amount of smooth muscle present in different organs in various animals, and partly to intrinsic differences in the degree of sensitivity which the same organ may acquire in various species.

9. Mechanism of Anaphylaxis. Antibodies

There can hardly be any doubt that the reaction of anaphylaxis is essentially the result of an antibody–antigen reaction. Evidence that antibodies play an essential role is overwhelming, and may be briefly summarized here: (*a*) only antigenic or haptenic substances will induce anaphylaxis; (*b*) the incubation period for active anaphylactic sensitivity is of similar length to that for antibody production; (*c*) the specificity of the reaction is exactly similar to that of other serological reactions; (*d*) specific desensitization of sensitive animals by injection of antigen is hard to explain except by assuming that antibodies present are temporarily saturated with antigen; (*e*) sensitivity can be passively conferred on a normal animal by transfer of serum from a sensitized animal; (*f*) by suitable technic (14), antibody can be demonstrated in the serum of sensitive animals; (*g*) the ability of a serum to confer anaphylaxis passively is approximately proportional to its precipitin content (28).

The failure of some workers to find precipitating antibodies in serum which would sensitize passively is probably due to failure to use a sufficiently delicate technic, for rather small amounts of antibody are enough to sensitize. Kabat and Landow (49) have reported that injection of 0.03 mg. of rabbit antibody nitrogen will sensitize a guinea pig sufficiently for fatal anaphylactic reaction, if the proper amount of antigen is used. This is not the "equivalent amount" as determined by the precipitin test, but corresponds rather to amounts found in the "inhibition zone" (page 233). An isolated guinea pig uterus will contract even though containing amounts of antibody not exceeding 0.01 microgram of antibody nitrogen. This reaction is therefore much more sensitive than the precipitin or even the complement fixation test. Also, it has been found that antibody which reacts demonstrably but does not precipitate may be present in some sera (see page 66). It is not impossible that such antibody might be able to sensitize.

10. Site of Anaphylactic Reaction

There have been two theories as to the place where the reaction causing anaphylaxis occurs. According to one view, the reaction occurs in the circulation and causes the production of toxic substances (humoral theory). According to the other view, the reaction occurs with antibodies present in, or fixed to, the tissues (cellular theory).

The available evidence is definitely in favor of the cellular theory, at least in so far as guinea pigs are concerned, and may be summarized thus: (*a*) An incubation period is almost always necessary for the development of passive sensitivity, suggesting that time is required for the introduced antibodies to become fixed in the tissues. If the reaction were purely a "humoral" one, there is no reason shock could not always be produced immediately after the transfer of the serum. It has been shown that during the incubation period a large proportion of the injected antibody does actually leave the circulation (101). (*b*) A sensitive animal can still be shocked readily even after its own blood has been replaced by that of a normal animal. (*c*) Possession of a large amount of circulating antibody actually tends to protect an animal against anaphylactic shock, probably by combining with antigen which would otherwise combine with "sessile" antibody in the sensitive tissues. (*d*) The smooth muscle of a sensitive animal, when removed and tested *in vitro*, even after thorough washing, reacts vigorously when brought in contact with the specific antigen.

It has been found (23, 82) that the intestine or uterus of a sensitized guinea pig, removed from the body and suspended in oxygenated Ringer's solution, will contract maximally when exposed even to very small amounts of the antigen. This has provided a valuable experimental technic for the study of anaphylaxis, allergy, and related problems (Schultz-Dale reaction).

11. Cause of the Reaction

Here, too, there have been two theories. One supposed that union of the antibody with the antigen disturbed the "colloidal equilibrium" of plasma or cell protoplasm, so interfering with the normal physiology of the tissues that shock is produced (physical theory). The other theory supposed that as a result of the antibody–antigen combination a toxic substance was produced which caused the shock (chemical theory). If we are willing to suppose that the toxic substance comes not from the antibody or antigen, but from the tissues themselves, it would seem that the chemical theory is better supported by the evidence (references in 87, 96), and it seems decidedly less vague.

From the work of Manwaring and associates it appears that in the dog during anaphylaxis there is liberated from the liver a toxic substance, which is responsible for many of the phenomena of shock. If the liver is removed, or excluded from the circulation, shock is prevented, and if the liver of a sensitive animal is "transplanted" to a normal dog, the latter can be shocked by the injection of antigen. A normal piece of intestine or bladder transplanted to a sensitive animal shows typical contraction when the latter is shocked.

It has been suggested (1, 63) that the toxic substance responsible for anaphylactic shock is histamine, or a histamine-like substance. Lewis (63) found that the local reddening and succeeding local wheal, following a local stimulus or injury to the skin, were independent of the local neurovascular mechanism. He concluded that they were the result of a chemical stimulus from some substance coming from the injured cells; and he found that, of the many substances he tested, only histamine would produce the same effects.

Other workers (6, 24, 40, 95) have demonstrated that histamine is actually a normal constituent of many tissues, not free, perhaps, but in loose combination with other cell components.

There is considerable evidence in favor of the histamine suggestion: (*a*) the susceptibility of a given species to anaphylactic shock usually parallels its susceptibility to histamine poisoning; (*b*) animals in which an increased tolerance to histamine has been built up by pretreatment are often more resistant to anaphylaxis (references in 50); (*c*) the tissues which play the most dramatic part in acute shock are those which are most sensitive to histamine; (*d*) formaldehyde, thymoxyethyldiethylamine, Pyribenzamine, and Benadryl, each of which has an antagonistic effect on the reactions produced by histamine (80), have a preventive action on anaphylaxis; (*e*) substances chemically and pharmacologically resembling histamine can be found in the circulation of animals during shock (5, 29, 30). Sherwood *et al.* (91) found that histamine produced a reaction in turtles which resembles the anaphylactic response.

Some observations which seem contrary to the histamine hypothesis have been cited, such as differences in the response of rat uterus to histamine, and behavior during anaphylaxis, although according to some workers (98) the two reactions *in vivo* are essentially the same. Campbell and Nicoll (13) found a substance to be released by guinea pig lung during anaphylactic shock which stimulated rat uterus, which is unaffected by ordinary doses of histamine. Went (104) considers the most likely hypothesis is that, during anaphylaxis, different biologically active substances are released from different tissues, depending on the chemical nature of the tissue. For example histamine might be liberated from the lung, choline from the heart, adrenaline from the adrenals, etc. Went obtained evidence that the isolated guinea pig heart behaves during anaphylaxis as if stimulated by a substance of the choline group, rather than histamine. He also believes that the pathological changes of anaphylaxis occur not only because of the increased liberation of histamine, choline, etc., but also in part because the shock organs of the sensitized animal are more susceptible to these substances.

It is worth noting that a number of workers have found that the enzyme histaminase does not protect against anaphylaxis (2, 77).

B. Hypersensitiveness in Man

I. SERUM SICKNESS

It is not easy to decide whether serum sickness is a form of anaphylaxis.

After the introduction of diphtheria antitoxin therapy in 1890, the injection of foreign serum became relatively common, and it was soon found that a portion of those receiving such serum developed unfavorable reactions, often leading to a curious and characteristic condition which was given the name of *serum sickness.* It was natural to suppose at first that the reaction was due to the antitoxin content of the serum, but it was observed that injection of normal serum could produce the same results. Indeed, it is probable that the reaction had been observed a number of years before, following transfusion of animal blood into man, but its nature was not then recognized.

1. Symptoms

The illness is characterized by the occurrence of rashes, especially of an urticarial type, which often commence at the site of the injection, by fever, pains in the joints, and glandular swelling, particularly in the lymph glands near the site of injection. This last may often be the first symptom noticed. There is slight edema, and there may be some albuminuria.

In persons not already sensitive to serum there is almost always an incubation period of 8 to 12 days between the injection of the serum and the onset of symptoms. In such persons, serum sickness, although it may produce severe discomfort, is probably not often fatal. In this respect it may be distinguished from *serum shock,* about to be discussed. It is characteristic of serum sickness that it can result from a single injection, even in individuals who appear to have no previous sensitivity to the proteins of the animal providing the serum. Recurrent reactions have also been observed (references in 19), again after only one injection, with intervals between the eruptions of as little as three to four, or as much as 19 to 21 days.

Pathological examination of fatal cases has sometimes shown cardiac damage.

2. Mechanism

An explanation of serum sickness which still seems the most plausible was proposed by von Pirquet and Schick (72). Their suggestion may be paraphrased thus: following the injection of foreign serum, the patient begins to form antibodies against certain of the serum proteins. After a time, these antibodies appear in amounts sufficient to react with the antigens responsible for their production, traces of which have persisted in the patient's circulation. This reaction is supposed to cause the symptoms. The mechanism would thus be quite similar to that of anaphylaxis, except that the latter is never observed to follow the primary injection.

In support of the idea of von Pirquet and Schick there are several observations: there is almost always a considerable incubation period before symptoms begin, which could be the time required for antibody production to get under way; the period of incubation may be shortened or be lacking in persons having previously had injections of the serum, or having naturally acquired sensitivity to it; and local edema sometimes occurs at the site of reinjection of serum, suggesting a local antibody–antigen reaction. Furthermore, as would be required by the suggested mechanism, antibodies reacting in various ways (precipitins, anaphylactic antibodies, heterophil antibodies—anti-sheep hemolysin and agglutinin—and serum reagins; see page 329) often appear in the blood of the affected individual, more usually after the onset of symptoms. This was thought to be due to a sudden throwing off into the circulation of antibodies which the cells have been gradually elaborating, probably at an increasing rate. The patient's skin is also likely to become specifically reactive.

If these ideas are accepted, the recurrent reactions referred to above could possibly be interpreted as due to the successive appearance of antibodies to various antigenic components of the serum (19, 25, 44).

Some doubt has been thrown on this theory, chiefly because precipitin may be found in the absence of serum disease, and serum disease may occur in the absence of circulating antibodies. It may be supposed, however, that the really important antibodies for this reaction are perhaps not those found in the circulation, but sessile antibodies attached to the cells. The appearance of the former might be merely an indicator of the presence of the latter, and the relative plentifulness of the two varieties of antibody would not necessarily run parallel. Coca (19), however, has also objected to the von Pirquet-Schick theory, and called attention to the similarity of serum sickness and drug allergy (see page 336), where antibodies have not been convincingly demonstrated.

Patients who have been previously treated with serum often exhibit a certain degree of sensitivity. Even small amounts of serum may be sufficient to produce sensitization. Hooker (45) and Tuft (97) found that, following the three injections at weekly intervals of the very small amount of horse serum (about 0.01 cc.) contained in diphtheria toxin–antitoxin mixtures then in use in diphtheria immunization, more than 25% of normal individuals became sensitive. If such individuals, or persons having naturally acquired sensitivity to proteins of the species from which the serum originates (e.g., horse asthmatics), are injected with serum, there may be a reaction which sets in sooner than in ordinary serum sickness (in some cases immediately), and which may be correspondingly more severe. In very severe cases the symptoms resemble those of anaphylaxis. This is called by clinicians "serum shock." Fatal cases are fortunately quite rare; 50 cases were reported up to 1930 (97). The pathological findings in such cases usually suggest those of guinea pig anaphylaxis, but at least one case has been reported where they resembled more those in the dog.

It is difficult to escape the impression that death in such human cases is essentially due to the same mechanism as that of anaphylactic death in animals. If we accept the idea that serum sickness is an example of the immunological mechanism gone wrong, we may assume that the more rapid appearance of symptoms is a sign of a better development of antibodies. Therefore, the earlier the symptoms, the more severe they tend to be, the more so since the amount of foreign antigen remaining to react with the induced antibodies is greater, as it diminishes constantly after the injection.

3. Prophylaxis

Serum sickness and serum shock are best avoided by not giving foreign serum to persons who are sensitive to it. Skin or eye tests should be made on all patients who are to receive serum. If no reaction is obtained, the physician has provided himself with pretty good (but not infallible) insurance that no fatal accident will follow administration of the serum. The tests should be done with normal serum of the species from which the antiserum originates (e.g., if horse antitoxin is to be given, test the skin with normal horse serum).

In addition, the patient should be carefully questioned concerning any previous administration of serum, whether for prophylactic or therapeutic purposes, and about immunization with diphtheria toxin–antitoxin mixture. (Note that immunization with toxoid, which contains no serum, will of course not sensitize to serum.) The patient should be questioned also about any previous allergic symptoms in himself or in his family, and particularly (if horse serum is to be used) about asthmatic sensitivity to horses.

As active immunization against the more common diseases becomes more widespread, and if chemotherapy makes the strides forward it promises to, we may anticipate that the seriousness of the problem will decrease, since therapeutic administration of serum will less often be necessary. Also, it seems that the serum of animals other than the horse is often less likely to cause serum sickness.

4. Desensitization

It will be remembered that the repeated administration of doses of antigen too small to shock will make an animal temporarily insusceptible to anaphylaxis with this antigen. A similar technic has frequently been tried with human patients who are sensitive to serum, by giving small, gradually increasing doses at intervals of about half an hour. However, there is no convincing clinical proof that such procedures are of definite value in preventing serious serum reactions. Particularly if a natural serum sensitivity exists, such methods are likely to be without value.

5. Treatment of Symptoms

The only treatment of any value consists in the administration of adrenaline subcutaneously or, if required, intravenously. If the serum has been given subcutaneously or intramuscularly in a region where a tourniquet can be applied above the site of injection, such application may be helpful in delaying the entrance of further serum into the circulation.

II. ALLERGY

The phenomena we are going to describe in the following sections differ from the foregoing in a number of respects. They have in common the characteristic that they have been observed chiefly in man. It is likely, however, that this difference is in part due to our preoccupation, in clinical work, with the human species. This fact may account for some of the novel features which will be observed. Many of the conditions may exist also in the lower animals, or could be produced under suitable experimental conditions. We cannot be certain, indeed we may well doubt, whether the reactions in other species would be in all respects the same. Some of the ways, therefore, in which human hypersensitiveness seems to differ from hypersensitive phenomena in animals may depend upon intrinsic species differences in modes of response. Perhaps the best definition of allergy would be: all forms of hypersensitiveness except anaphylaxis. Von Pirquet (71) coined the word allergy, meaning literally "changed or altered reactivity," to denote any acquired specific alteration in the capacity to react which occurs in living organisms or tissues upon exposure to living agents or inanimate substances. As originally framed, this definition was meant to include alterations in the direction of protection as well as in the direction of hypersensitivity, so that, strictly, immunity would also be connoted by the word. Von Pirquet felt that a single word was desirable to embrace all these different specific alterations in reactivity since he felt, as we do, that they were all fundamentally related biological phenomena. However, it has not proved convenient in practice to use a word which is, as it were, two-edged, so that if an animal is said to be allergic to an agent it is not known whether he is more or less resistant. The word allergy, therefore, although never authoritatively redefined, has in actual usage lost the connotation of immunity, and now implies what we have above expressed as hypersensitiveness, except that it is not usually used to denote anaphylaxis. Different authorities have not always agreed in their use of the word, and it has finally found its way, usually in an incorrect sense, into the popular press, so that it is not easy to know precisely just what a given person means by allergy. We will attempt to escape from this difficulty by subdividing allergy into a number of types, which we may hope to define more precisely, and discuss one by one.

The word allergen, analogous to antigen, denotes the excitant of an allergic reaction. Allergens include some substances which are not antigenic in the ordinary sense. According to the route through which they produce their effects, they may be classified as inhalants, ingestants, contactants, injectants, and physical and nonspecific factors. Examples are pollens, dusts, danders, foods and drugs, poison ivy, formaldehyde, arsphenamine, bacteria, molds and fungi, and apparently also agents such as heat, cold, and sunlight.

The chemistry of allergy has been reviewed by Newell (69) and by Coulson *et al.* (22). The allergen in pollen is reported to have a molecular weight of about 5,000.

1. DIFFERENCES BETWEEN ANAPHYLAXIS AND ALLERGY

We have refrained from attempting to classify serum sickness exactly. The remaining topics in this chapter will be included under allergy. A number of authors have included anaphylaxis also under allergy, and certain important similarities between them will be noted in perusing the following sections, but there are differences which are sufficiently marked to warrant classifying the two conditions under separate headings. We may tabulate some of the most striking of these in the following scheme, modified from Tuft (97) (Table XL).

TABLE XL

DIFFERENCES BETWEEN ANAPHYLAXIS AND ALLERGY[a]

	Anaphylaxis	Allergy
Occurrence.	Artificially induced.	Usually naturally acquired.
Hereditary factor.	Not yet reported. (Congenital passive sensitization possible by passage through placenta).	Often present; predisposition can be inherited through either mother or father (rarely congenital).
Duration of sensitization	Relatively brief.	Long.
Nature of antigen.	Usually protein.[b]	Induced by many non-protein substances.
Characteristic antibodies.	Precipitins.	Reagins[c] (when present).
Symptoms.	Due to contraction of smooth muscle. Same irrespective of type of antigen.	Usually due to edema, but some reports indicating smooth muscle contraction. May differ with different antigens.
Pathological findings.	Shock organ variable in different species but constant in individual members (see p. 316).	Shock organ may differ in different individuals; even in same individual more than one may be involved.
Desensitization.	Relatively easy, at least in the guinea pig.	Difficult to produce, or to prove.

[a] Modified from Tuft (97).

[b] Landsteiner and Jacobs (59) have reported anaphylactic sensitization to arsphenamine. However Landsteiner (55) favored retaining the distinction between allergens and antigens, reserving the latter term for substances of high molecular weight.

[c] See text, page 329.

2. HEREDITARY FACTOR

Observations indicating the existence of a hereditary predisposition, both in hay fever and in asthma, were made long ago. Modern work has established it practically beyond question. Details may be found in (19, 97). The hereditary factor does not seem to be present in all forms of allergy, however, and Coca has proposed the designation of *atopy* for the forms where a definite hereditary basis has been found.

Wiener, Zieve, and Fries (105) have suggested that the predisposition to allergic disease may be transmitted by means of a single pair of allelomorphic genes, H and h, in which H determines nonallergy, and h determines allergy. Three different genotypes are possible: HH, pure normal; hh, allergic individuals whose symptoms begin before puberty; and Hh, individuals who may be normal transmitters, or develop allergic disease after puberty.

It has been shown that a hereditary factor operates in the susceptibility of guinea pigs to sensitization (47).

3. Importance of Contact

It will of course be realized that the role of heredity in these conditions is simply to predispose the affected individual to sensitization. The hereditary predisposition alone is not enough to make the individual actually sensitive; contact with the active agent (called the *allergen*, by analogy with antigen) is also necessary. This is well shown by experiments by Grove (cited in 19) who tested 35 patients with timothy hay fever residing in Berlin, where there is no ragweed. None of these patients had been in the United States, where ragweed is common. Grove found none of the 35 to react even to strong extracts of ragweed. Since about half the timothy hay fever patients in the United States are also subject to ragweed pollen hay fever, about 17 of the Berlin cases were, as Coca points out, *potentially* ragweed sensitive, so that if the hereditary predisposition alone were sufficient, they should have reacted. This is supported by Grove's test of another timothy hay fever sufferer in Berlin who had been to the United States. This individual reacted to the ragweed pollen extract.

Contact is necessary, therefore, to make an individual sensitive, no matter what his heredity. It is evidently not sufficient, however, for in the types of allergy where the role of heredity is clearest, it has been found that even extensive and prolonged contact with an allergen will not render a normal individual sensitive, at least not to the same degree as one predisposed to sensitivity (10, 32). Figley and Elrod (32), who traced an endemic focus of asthma to dust from a castor oil mill, consider that their study "apparently refutes Van Leeuwen's contention that asthma can occur in normal persons when the factors of irritation and prolonged contact are present."

In types of hypersensitiveness where the role of heredity is less marked, apparently almost every individual can be sensitized by adequate contact. This is clearly suggested by the results of the administration of even small amounts of horse serum, already referred to on page 324.

4. Allergic Antibodies (Reagins)

In a number of allergic conditions antibodies can be demonstrated in the patient's serum. The behavior of these antibodies differs in a number of respects from that of ordinary antibodies, and some authors prefer to use the noncommittal term reagin. The chief differences between reagins and anaphylactic antibodies are shown in Table XLI.

Although neutralizing antibodies have been found (see 64), it will be noted that *reagins* do not precipitate or neutralize the antigen (called allergen or "atopen"). They may however be detected by their power of passively sensitizing the human skin. If a small amount (0.1 cc.) of the allergic patient's serum is injected into the skin of a normal individual, after a suitable interval (24 to 48 hours) the prepared site in the normal will now react (by reddening, itching, and edema) when injected with a small amount (0.02 cc.) of the specific excitant, or when the excitant is introduced into the blood, although untreated sites on this person's skin still react negatively. It is said that the skin of a small number of normal individuals can not be thus sensitized, this demonstration of reagins being successful only in the skin of the larger "receptive" group.

Loveless (64) injects a mixture of reagin and antigen and reads the immediate reaction which results.

Sensitization may also result from the transfusion of blood from a sensitive person into a normal (see 65).

Other peculiarities of reagins brought out in Table XLI are their inability to sensitize the guinea pig uterus, and their failure to neutralize the antigen. This results in a peculiar nonreciprocal interrelation of allergen and reagin. It is found that, if a mixture of egg white and serum (from an egg-sensitive patient) which contains reagins to egg white is incubated and injected into a site previously passively sensitized to egg, a positive reaction will be obtained, indicating that the inciting power of the antigen has not been destroyed. But at the same time it is claimed that such a mixture will not sensitize the skin of a normal person, suggesting that the sensitizing power of the reagin in the mixture has been lost. Loveless (64) found that reagins to ragweed were never produced in nonsensitive subjects injected with ragweed extract, although the reagin titer of sensitive subjects was usually increased by such injections.

It is unknown what physical and chemical differences account for the special behavior of the reagins. Nothing is known about the actual amounts of reagin present in the serum of sensitive persons, and one reason for their failure to precipitate, etc., as ordinary antibodies do, might be that they are present in very minute amounts. Kabat and Landow (49), studying passive anaphylaxis, obtained evidence which led them to suggest that only about 0.01 microgram of antibody nitrogen might be sufficient to sensitize the area of skin used for the usual passive transfer. The complement fixation test could detect not less than about 20 times this, and the agglutination and precipitation tests are even less sensitive. More or less contrary to this, however, is the observation that very small amounts of allergic serum may sensitize a normal skin so highly that it will react with extremely small amounts (dilutions of over 1:10,000,000) of the antigen, and that some sera can be diluted (about 1:100–1000) and still sensitize.

TABLE XLI

COMPARISON OF REAGINS WITH ANAPHYLACTIC ANTIBODIES[a]

Reagins	Anaphylactic antibodies
Sensitize human skin, often in very small quantity.	Do not sensitize the human skin.
Quickly and permanently attached to body cells.	Diffuse from injection site through the body.
Do not sensitize the guinea pig.	Sensitize the guinea pig.
Not precipitating antibodies.	Probably precipitating antibodies.
Typically do not neutralize the antigen.	Neutralize the antigen.
Heat labile (largely destroyed, half-hour at 56°C.).	Heat stable (slightly affected, half-hour at 56 °C.).

[a] Slightly modified from Coca (19).

Antibodies possessing, like reagins, the power of passively sensitizing the human skin have been observed in the sera of rabbits and guinea pigs injected with pollen extracts (89, 106). This property of the sera did not appear to be correlated with the precipitin content; in most of the guinea pig sera no precipitins were found.

Specific reagins are not found in all allergic individuals. They are usually found in subjects with hay fever, asthma, and some kinds of eczema. They do not yet seem to have been demonstrated in urticaria, angioneurotic edema, tuberculin hypersensitiveness, drug allergy, or contact dermatitis. Reagins have not been found except in patients in whom a positive skin reaction to the allergen can be demonstrated, but they may be absent in such patients.

The concentration of reagin in the blood seems to be proportional to the degree of skin sensitivity, but does not necessarily parallel the degree of clinical sensitivity (severity of symptoms). It has been reported that the titer of demonstrable antibodies in various organs correlates well with the occurrence of allergic reactions (35).

"Reversed" skin reactions (injection of the antigen into the skin, followed by injection of the serum of a sensitive individual) have been reported (108). This is reminiscent of "reversed passive anaphylaxis" (page 313).

5. ATOPY

The term atopy, meaning "a strange disease," was proposed by Coca to denote certain clinical forms of human hypersensitiveness which are affected by a hereditary predisposition. It was once supposed that this condition did not occur in animals, but since that time it has been observed in dogs (11, 73, 100), cattle (7), and other animals (9, 16, 46, 107), including a walrus (81).

Hay fever and asthma are classified under atopy; it is likely that we should include here most forms of infantile eczema, and certain forms of drug and food idiosyncrasy. The hereditary factor involved in atopy has been discussed on page 327. For the individuals inheriting the tendency towards atopic allergy, a slight degree of contact is sufficient to produce sensitivity. There is evidence that other "normal" individuals may, if continuously exposed to allergens, as in the course of their daily work, acquire some degree of sensitivity. Thus 30 to 40% of bakers give skin reactions to rye, wheat, or other grains, and over 20% of cavalrymen react to horse dander (see 43). Few such persons have clinical symptoms. Even where sensitivity is induced by prolonged or intimate contact (as by the injection of serum), the sensitivity produced is likely to differ from atopic hypersensitivity. This is shown in Table XLII.

The distinction between the two types of allergy exemplified in Table XLII is not absolutely sharp, but has been found useful in clinical practice. The differences are most striking when allergies of the definite atopic type are compared with such typical acquired allergies as contact dermatitis and allergy of infection (tuberculin type) (see Chapter IX). There are a number of fairly common allergies which may be of either type; an example is provided by the different degrees of serum sensitivity exhibited by horse asthmatics and by persons rendered sensitive by previous injections of serum.

TABLE XLII

DIFFERENCES BETWEEN "NATURAL" (ATOPIC) AND NONATOPIC TYPES OF HUMAN HYPERSENSITIVENESS*

Characteristic	Atopic	Nonatopic
Degree of sensitiveness.	Often very high.	In many cases less.
Severity of symptoms after injection of allergen.	Likely to be severe or even fatal.	Usually less intense.
"Desensitization."	Difficult to accomplish, even partially.	Often sufficiently successful to permit introduction of required amount of allergen.
Reagin (allergic antibody).	Often in large amount, permanent, little influenced by treatment.	Small amount, temporary, or not demonstrable.
Skin or eye test.	Usually specific; often strong.	More often negative, when positive, relatively weak.
Association with other allergic conditions.	Comparatively frequent.	Infrequent.
Role of heredity.	Definite.	Less pronounced.

* Modified from Tuft (97).

Other allergies which may be of either type are drug allergies, physical allergy, food allergy, allergic rhinitis, urticaria, and angioneurotic edema.

6. MECHANISM

Just as there is good reason to think that anaphylaxis is an antibody-antigen reaction, so there is persuasive evidence that the reaction in the natural or atopic type of allergy is also a reaction between the allergen and an antibody, which in this case is called reagin. We may cite two main facts: (*a*) individuals with the atopic types of hypersensitiveness, such as hay fever, asthma, and eczema, have reagins in their blood. These reagins are specific for the allergen and their concentration usually parallels the degree of skin sensitivity. (*b*) The reagin content of the blood is observed to increase to from two to four times its initial level following injection of pollen extracts, suggesting that the reagins are produced in response to the antigenic stimulus of the allergen.

It is also believed, again in analogy with anaphylaxis, that the allergic reaction is due to the combination of allergen with reagin in the tissues, and the immediate cause of the symptoms is believed to be the resulting liberation of histamine (51) or a histamine-like substance. It is probably the sessile or attached antibodies which are important here, for it is found that the sensitivity which can be transferred passively to a normal skin by injection of serum from a sensitive subject remains localized instead of diffusing as it would if it were due to circulating antibody.

The differences in the symptoms of different allergic reactions in the same species appear to be due largely to the localization of the process in one or more of the "shock organs." The important sites of such reactions may be conveniently classified into: conjunctiva, nasal mucosa, bronchi, skin, gastrointestinal mucosa, and the nervous system (brain). Clarke, Donnally, and Coca (17) have presented evidence indicating that the localization of the sensitivity is at least in some cases also determined by inheritance. The influence of other factors, such as the natural portal of entry of the allergen, is still in need of exploration.

III. TYPICAL ALLERGIC CONDITIONS

1. "Hay" Fever

The seasonal paroxysmal attacks of coryza, known as hay fever, are too familiar to require any extended description. The symptoms may be produced by pollens or mold spores, or both, in the air coming in contact with the upper respiratory membranes. The pollens come chiefly from three groups of plants, called popularly trees, grasses, and weeds. In our climate the trees pollinate early in the spring; symptoms due to them are likely to come in April, May, and June, but certain trees, particularly in the southern states, produce symptoms in the fall. Grasses bloom from late April to late July. In the United States weeds are the most important cause of trouble, trees the least. Since ragweed produces pollen so profusely and the plant is so widespread, it produces symptoms in a large number of people. It pollinates from about the first week in August to the first week in October. Although hay fever is usually caused by pollens, similar symptoms may be produced by other agents, such as house dust, orris root (a constituent of many cosmetics), and danders.

There can be little doubt that hay fever is due to an "immunization that does not immunize," in other words a sensitization of the susceptible individual by antigenic constituents of the pollen or other incitant. It has been demonstrated that pollen is antigenic for lower animals. Reagins are found in the blood of hay fever patients, usually in relatively large amounts. Pollen extracts produce marked skin reactions in sensitive subjects, and this is believed to be due to the combination between the pollen antigen and its specific reagin.

Since the pollen antigen is not neutralized by the reagin, as is shown by passive transfer (see page 329), but seems to be held in some sort of loose combination, it is apparent that "desensitization" (see page 341), by injection of pollen extracts, does not operate by neutralizing the reagin; it has in fact been found experimentally that the reagins in such cases are not neutralized.

As would be expected from our knowledge of specificity (see Chapter III), overlapping reactions are obtained with the pollens of closely related plants. Some allergists have advocated making use of this fact by using an extract of only one member of a plant group (instead of a mixture of the individual members) for "desensitization."

2. Asthma

This term designates a recurrent, periodic, or paroxysmal type of breathlessness or dyspnea. The symptoms include wheezing or whistling respiration, with prolongation of the expiratory phase. This is caused by obstruction of the smaller bronchioles, due to spasm of the bronchial muscles or swelling of the mucosal lining, or both. It is not always caused by allergic factors, and the term asthma is really only a name for a symptom complex. Depending on the site of the primary reaction, asthma may be subdivided into bronchial, cardiac, and thymic. Bronchial asthma may be further subdivided into allergic and nonallergic types. Of the bronchial asthmas, we shall consider here only the allergic type, which includes by far the greatest number of patients.

Allergic asthma may be produced by a variety of allergens; of these the most important are inhalants and ingestants. Asthma caused by inhalants may be regarded as essentially the same thing as hay fever, except for the differences in the location of the susceptible tissue (shock organ). It may be due to house dust, orris root, pollens, animal danders (such as dust from feathers, or epithelium from horses, cats, and dogs), glue in furniture, book bindings, straw hats, etc.

Ingestants causing asthma include foods and drugs. Foods are particularly important in infants or young children; older persons seem to develop an increasingly greater tolerance to foods, but become more frequently sensitive to inhalants. A large number of different foods, especially wheat, eggs, and milk, may be allergenic for particular individuals. Drugs may cause asthma by ingestion (e.g., aspirin, quinine, antipyrine, and members of the morphine group) or by inhalation, as in pharmacists and laboratory workers (ipecac, *p*-phenylenediamine, lycopodium, urease, peptone, etc.).

It is generally agreed that the shock organ in allergic asthma is the lining or the musculature of the bronchi. When the allergen comes in contact with the sensitive cells, the reaction liberates histamine or a histamine-like substance, which is responsible for the attack. Asthma is a much more crippling condition than hay fever, although patients rarely die even during an acute asthmatic attack.

3. Atopic Dermatitis

When the skin is the shock organ in atopy, the picture produced may vary in infants and adults, presumably due to differences in the skin at various ages. In the infant the result is a noninfectious, erythematous and itching, scaly, and vesicular or oozing dermatitis, which is called "eczema" by most physicians. The word eczema is however used to denote a heterogeneous collection of inflammatory dermatoses of the infant and young child, and is not a proper disease entity. If atopic eczema becomes chronic and loses its vesicular characteristics later in childhood, or in adult life, it may be designated by a number of different names, such as "prurigo" or "disseminate neurodermatitis" (94).

The excitants of atopic dermatitis are mostly foods, but may include some inhalants such as dust, silk, orris root, and animal danders. In infants egg, milk, and wheat, probably in about this order, are the most important.

Patients with atopic dermatitis often give positive skin tests to one or more (sometimes to a great number) of allergens. Reagins can often be demonstrated in such persons.

4. Urticaria (Hives)

This condition, sometimes also known as nettle rash, is an edematous or inflammatory disorder of the skin. Whitish, pinkish or reddish elevated spots, known as wheals or hives, appear, accompanied by an itchy or burning sensation. They tend to come and go, even within a period of an hour or less. There seems to be some evidence of a hereditary factor, but the condition is usually classified as nonatopic. The most frequent cause is probably allergy to foods; the frequency with which the condition results from eating strawberries is known to everyone. Drugs are probably also responsible in some cases, particularly phenolphthalein, which is a constituent of many proprietary laxatives.

Skin tests may help detect the specific excitant, but often fail in conditions due to food allergy. It is sometimes possible to determine the cause from the history of the complaint. Removal of the specific allergen or allergens in nonpsychogenic cases usually brings relief.

5. Angioneurotic Edema

This is similar to urticaria, but the lesions are large, pale swellings which may involve whole areas, such as the lip, eyelid, genitals, or even the side of the face or a whole hand. The edema involves the deeper layers of the corium and subcutaneous tissues. The condition has been called "giant urticaria." It may occur in association with other allergic diseases, and the excitant in such cases is usually the same. The diagnosis and treatment are practically the same as for urticaria.

6. Allergy to Simple Chemical Compounds (Drug Allergy)

There are two forms of increased susceptibility to drugs. In one type the patient is abnormally susceptible to the action of the drug, and its administration produces symptoms which are an exaggeration of its ordinary pharmacological action; in the other type the administration of the drug, in an amount nontoxic or perhaps even inactive for the normal individual, produces a reaction not characteristic of the drug, but of allergic conditions, such as fever, skin eruption, and pruritis. The second kind of sensitivity is what we mean by drug allergy. It is specific in most individuals, in the sense that the sensitivity exists to only one substance or group of chemically related substances. The symptoms show the same independence of the nature of the exciting agent which we have noted in anaphylaxis and other kinds of allergy.

The possible excitants of drug allergy include alkaloids, such as opium products and quinine, essential oils and balsams, such as turpentine and urushiol (the active constituent of poison ivy, 52), metals, such as mercury and arsenic (including some forms of sensitivity to arsphenamine), the halogens, various synthetic drugs (formerly called "coal tar derivatives"), such as antipyrine, salicylic acid, and arsphenamine, drugs of the barbituric acid series, and endocrine and other glandular products. Some asthmatics are atopically sensitive to aspirin.

In some cases the patient's reaction is caused, not by the whole drug molecule, but by a portion of it, or by some chemical group contained in it. In allergy to arsphenamine it has been found that some patients are sensitive to the organic part of the molecule, while others react to the arsenic. It has been claimed that iodoform sensitivity may be directed towards either the methyl radical or the iodine. It was found by Dawson, Sanders, and Tomlinson (26) that individuals allergic to quinine could be separated into several groups, depending on which part of the molecule was responsible for the sensitivity (see 92).

An interesting feature of drug allergy is that nearly all of the incitants are substances which are not antigenic. If we are to suppose that the fundamental mechanism is the same as in the forms of allergy already discussed, the problem arises, how can a nonantigenic agent sensitize? Following the publication of the work of Landsteiner and his co-workers (see Chapters II and III), which showed that it was possible to render simple chemical compounds antigenic (or haptenic) by attaching them chemically to proteins, it was apparent that a possible clue to this mystery had been found. It might be that allergenic drugs become so only by combining with the susceptible individual's own proteins. This suggestion had in fact been made previously, but without sufficient evidence.

There is some reason to think that drugs may be able to attach themselves to proteins in the body (see 25a). In some cases it is easy for a chemist to see how this might occur; in others it must be supposed that the attachment takes place after chemical alteration of the drug in the body. In fact Mayer (68), studying sensitivity to azo dyes and anilin derivatives, found various group reactions, not based on the complete original substances of exposure, but related to new derivatives formed in the body by oxidation, reduction, etc. An argument in favor of the formation of conjugates comes from the work of Landsteiner and Jacobs (59) who studied chlor- and nitro-substitution products of benzene. Treatment with sodium ethylate or sodium methylate was found to remove a chlorine atom or a nitro group much more readily (indicating greater chemical reactivity) from those substances which would sensitize guinea pig skin than from the nonsensitizers. It was also found that the sensitizing substances alone gave stable substitution compounds with aniline. Later, Landsteiner and Chase (57, 58) succeeded in sensitizing the skin of guinea pigs to a simple compound (picryl chloride) by injections of a conjugate of this substance with guinea pig stromata.

Although this seems to be a possible mechanism for the sensitization to drugs, it will of course be realized that its operation has not actually been demonstrated in the majority of cases, and the situation is such that the demonstration would in many instances be very hard. In addition, there remains a difficulty. So far, no one has demonstrated the presence of any sort of antibodies in drug allergy, except anaphylactic antibodies after treatment with picryl chloride (57). This does not of course prove that antibodies are not present, as our methods of testing for them may be inefficient, or they may be almost completely confined to the tissues (sessile). Even skin tests, except in eczematous drug eruptions, are as a rule of little or no value in determining the excitant in drug allergy (94).

There is a second, perhaps more common, type of drug allergy, in which the symptoms usually appear after an incubation period, as in serum sickness. As a rule the first administration of the drug produces no symptoms, so it may be considered that the sensitization is produced by the first dose or doses. The sensitivity, and consequently the immediacy of the reaction, may increase with repeated contact. The characteristic symptoms are fever and skin eruptions. The treatment consists in eliminating contact with the drug.

In experimental allergy to simple compounds, where animals are used, satisfactory sensitization is usually produced only by treatment of the skin, except under special conditions.

There are some observations which suggest that sensitization to simple compounds is more likely to result when they are applied to an injured area, as the sensitization to picric acid which occasionally results from the use of Butesin Picrate on burns. Also, Landsteiner and Di Somma (62) found that picric acid, which alone did not sensitize guinea pig skin, would do so when painted on after the application of cantharidin.

7. "Familial Nonreaginic Food Allergy"

Coca (18) has proposed a new category of allergies under the above name (the incitants are not merely foods, however, but are thought to include metals, drugs, etc.). He states that it differs from atopy in that (*a*) it is controlled by a different hereditary mechanism, (*b*) reagins are not found, (*c*) many of the symptoms are not found in atopy, and (*d*) the reaction practically always causes pulse acceleration.

8. Contact Dermatitis

This is an inflammatory disease of the skin, caused by external irritants which are harmless to the individual on first contact (before sensitization). The lesions are localized primarily in the epidermis and are of the vesicular type. In Europe the condition is usually called "eczema," and many American dermatologists call it "eczematous dermatitis." When caused by hypersensitiveness to such plant poisons as poison ivy, poison oak, and primrose, it is often called "dermatitis venenata." This name is not so appropriate today, since we know that these agents are not toxic on first contact, but affect only individuals who have been sensitized, although nearly all individuals can be sensitized, if the contact is sufficiently prolonged.

A very large number of substances can cause contact dermatitis. Many of these are substances with which the individual comes in constant contact in his occupation, and which produce the condition called occupational dermatitis, or trade eczema. This long list includes such substances as dyes, soaps, lacquers, woods, glue, rubber, drugs, metals, and explosives. In addition to the well-recognized poisonous plants, many others, including grasses and common weeds, may produce dermatitis. In many cases this seems to be due to an oily constituent of the pollen.

It has been shown that this condition is a true allergy, as the exciting substances are harmless to any individual on first contact. Thus very young children, and Eskimos (42), who have never had contact with poison ivy, do not respond to the direct application of it to the skin. It is customary to consider contact dermatitis as nonatopic, since almost any individual may be sensitized, although in varying degree. The difference might perhaps lie in the mechanism of inheritance; the inheritance of atopy might depend on one or at most a few genes, while the hereditary influences in the nonatopic conditions might depend on larger numbers of genes, resulting in more intermediate types and less sharp distinction between the normal and the susceptible individual.

In this disease too, "desensitization" has been tried, with some success, particularly in the case of poison ivy. In most cases, of course, avoidance of the excitant is easier.

9. Migraine

This is a periodic, incapacitating type of headache, usually unilateral. It may be accompanied or preceded by sensory disturbances, and may end in nausea and vomiting. The layman knows it as bilious headache, sick headache, or blind headache. The symptoms seem to be due to cerebral vasodilatation. There is indication that some forms of migraine may be allergic in nature. The hereditary factor is undoubtedly present, and some food occasionally seems to be the exciting factor. If the allergic hypothesis is correct, it may be supposed that the basic cause of such types of migraine is the same as in any other type of allergy, i.e., local edema resulting from capillary vasodilation and capillary permeability. An interesting observation tending to support this has been presented by Goltman (38, see 93).

10. Allergy of Infection (Bacterial Allergy)

This subject will be discussed in the following chapter, in connection with immunity to tuberculosis and to virus and other diseases.

11. Treatment of Atopic Allergy

Three basic measures are possible in the treatment of the above conditions: (*a*) we may have the patient avoid or eliminate, if possible, the excitant or excitants; (*b*) we may attempt to remove or lessen the degree of susceptibility by "desensitization;" (*c*) in acute attacks, or where the specific excitant can not be discovered, symptomatic treatment (e.g., adrenaline) may be given.

(*a*) Elimination or avoidance of the excitant is always the preferable method, but may be difficult or impossible if the patient is sensitive to a large number of substances, or if his occupation requires his presence in places where the excitant can not be avoided. In any case the method presupposes that the specific excitant is known, or can be discovered (skin tests, etc.). If the sensitivity is to a single allergen which can be completely avoided, the results are completely satisfactory.

(*b*) Desensitization might be spoken of as lessening of sensitization, or the term hyposensitization proposed by Cooke and Coca might be used. The process may really be an immunization (65). Desensitization in allergic conditions differs from desensitization in anaphylaxis; it is rarely complete, and in allergy desensitization does not lessen the antibody content of the blood. Nevertheless, the procedure has considerable value, for it lessens or even abolishes (temporarily) the sensitization of some patients. Desensitization may be specific or nonspecific. The latter term refers to the use of agents unrelated to the excitant, such as bacterial vaccines, peptone, and tuberculin. The mechanism of this procedure, if it is really very effective, is as yet unexplained.

Specific desensitization consists of the introduction of very small amounts of the specific allergen at frequent intervals, until the clinical susceptibility is diminished. Usually the dose is gradually increased. The first dose may be roughly gaged by the degree of skin reactivity to the allergen. Desensitization to ingestants is not often necessary, as the patient can avoid the food or drug. If it should be necessary, the administration by mouth of successively increasing amounts of the allergenic food is the method to be preferred.

Specific desensitization is most often undertaken in the case of allergy to inhalants. It is done by injection subcutaneously of an extract of the allergen. The inhalants most frequently employed are pollens, molds, dust, and animal danders.

Loveless (64) has pointed out that following the injection of pollen extracts into pollen-sensitive patients three changes occur: thermostable antibody is produced in the serum; a relative immunity to the administered antigen is achieved, as reflected in the behavior of the conjunctiva and skin; and clinical benefits result. She suggests that all three may be referable to one mechanism, which is production of antibody which has a binding action on the antigen. Sherman (88) also thinks this "blocking" antibody is an important factor and Cooke (20) believes it significant. This interpretation is not accepted by all workers, however (86).

The thermostable antibody produced differs from reagin (which may be produced in increased amounts also, in naturally sensitive patients) in that it resists heating to 56°C. for five hours, or 60° for half an hour, and has the power of combining with antigen in the tissues, without causing the irritation which follows combination of reagin and antigen. If present, it will "block" (21) the reagin, so that, if this antibody, reagin, and antigen are introduced into the skin, no urticarial reaction follows, unless more antigen is present than the blocking antibody is capable of combining with. Hampton *et al.* (39) report that the thermostable antibody will also block rabbit antiragweed precipitating antibodies and that the method compares favorably with the passive transfer procedure. Since the tests with rabbit antibody are carried out entirely *in vitro*, this makes the new method potentially preferable.

Lowell (66, 67) has reported finding in the serum of an insulin-resistant patient two antibodies; one a heat-labile allergic antibody capable of conferring sensitivity on normal skins, the other a heat-stable neutralizing antibody, capable of preventing the normal physiological action of insulin.

(*c*) Many patients require drug therapy at some time. Four groups of drugs, exemplified by adrenaline, atropine, morphine, and a newer group of synthetic antihistamine substances, such as Pyribenzamine and Benadryl (37a, 67a), have been used. Of these adrenaline is the most useful, chiefly because of its immediate action. Ephedrine has a similar effect, but takes longer to act; it has a more prolonged effect however. Atropine and similar drugs are helpful in asthma because of their action in dilating and drying the respiratory passages.

SUMMARY

Immunology treats not only of mechanisms which increase resistance, but of some, similar in mechanism, which result in decreased resistance, such as hypersusceptibility. These phenomena are difficult to classify, but the terms anaphylaxis and allergy can be used to include nearly all, or perhaps all of them. (*A*) *Anaphylaxis.* Anaphylaxis is the opposite of prophylaxis, and denotes a lowering of resistance; it is not necessary, however, for the inciting substance to be toxic in itself. (*1*) Animals can be sensitized to anaphylaxis by most antigens. Extremely small amounts of antigen may suffice. Some haptens have been observed to sensitize. An interval of two weeks or so must elapse before maximum sensitivity results. (*2*)

Anaphylactic shock in a sensitized animal results when antigen comes suddenly in contact with the sensitized tissues; therefore injection into the circulation is most effective. The dose of antigen should be larger than the sensitizing dose, but not too large. (*3*) If the shocking dose does not kill, the animal is refractory to shock (by that antigen) for a short time. Animals may be desensitized deliberately by slow introduction of the antigen, or by successive small doses. (*4*) Anesthesia, or certain other treatments, may sometimes lower reactivity nonspecifically. (*5*) Anaphylaxis exhibits about the same degree of specificity as do other serological reactions. (*6*) A normal animal may often be made susceptible to shock by the injection of serum from a sensitized animal, even one of a different species (passive anaphylaxis). Usually an interval must elapse after the injection of serum. (*7*) The symptoms of anaphylactic shock vary in different species; in the guinea pig signs of respiratory distress come first, followed by cyanosis, defecation, and urination. The body temperature falls. In animals other than rodents nausea and vomiting are prominent symptoms. (*8*) The pathological changes of anaphylaxis have been traced by some workers to contraction of smooth muscle, and increased capillary permeability. In the guinea pig and the rabbit the immediate cause of death is probably contraction of smooth muscle in the bronchi and in the pulmonary arterial system, respectively. (*9*) The mechanism of anaphylaxis is considered to be the combination of antibody and antigen, resulting in a toxic effect. Evidence that antibodies (not necessarily circulating) play a role is overwhelming. (*10*) It was once thought that the reaction occurred in the circulation, but modern workers feel that it occurs (in the guinea pig, at least) with antibodies present in, or fixed to, the tissues (cellular theory). Isolated tissues (e.g., guinea pig uterus) will react *in vitro*. (*11*) There is evidence that the antibody–antigen reaction liberates a toxic substance which is responsible for the shock. It has been suggested that this substance is histamine, but some workers doubt this, or feel that other substances may sometimes account for the symptoms.

(*B*) *Hypersensitiveness in Man.* (*I*) Serum sickness is somewhat similar to anaphylaxis, but results from one large dose of foreign serum. (*1*) The symptoms are rashes, fever, edema, etc. (*2*) It has been suggested that the mechanism is the reaction of antibodies produced in response to constituents of the foreign serum with some of its constituents still remaining when the antibodies are released.

This would account for the latent period of a week or more which is observed. If foreign serum is given to persons who have been sensitized (by previous injection or otherwise as in asthma) a reaction like serum sickness, but setting in sooner, perhaps immediately, may result. In severe cases the symptoms may resemble those of anaphylaxis. (*3*) Serum sickness and serum shock are best avoided by not giving foreign serum to actually or potentially sensitive individuals. Skin tests and a careful history, including history of allergic disease in relatives, help in making the decision. (*4*) The evidence is not convincing that desensitization to foreign serum, by the administration of small, gradually increasing doses, has any definite value in preventing serious serum reactions. (*5*) The only treatment seems to consist in the administration of adrenaline (or similar drugs). (*II*) Allergy originally meant "changed or altered reactivity," but in modern usage generally denotes hypersensitivity. Allergens, analogous to antigens, are the excitants of allergies. Classified by route they can be inhalants, ingestants, contactants, etc. They may be pollens, dusts, danders, foods, drugs, etc. (*1*) The chief differences between allergy and anaphylaxis are: (*a*) allergy is heritable, anaphylaxis is not; (*b*) allergy is naturally acquired, anaphylaxis is artificially induced; (*c*) allergic individuals remain sensitive for a long time, strong anaphylactic sensitivity is of shorter duration; (*d*) allergy is often due to nonprotein excitants, anaphylaxis has been thus produced but seldom; (*e*) allergy symptoms are generally due to edema, anaphylaxis to smooth muscle spasm; (*f*) it is difficult to desensitize in allergy, but relatively easy in anaphylactic sensitivity; (*g*) allergic antibodies (when present) are *reagins*, anaphylactic antibodies are precipitins. (*2*) A hereditary factor operates in at least some allergic conditions. (*3*) Even if the hereditary predisposition is present, however, contact with the excitant (allergen) is required to produce sensitivity. (*4*) In a number of allergic conditions antibodies can be demonstrated in the patient's serum. These antibodies (reagins) differ from typical antibodies (such as anaphylactic antibodies) since they: (*a*) sensitize the human skin, (*b*) are quickly and permanently attached to the body cells, (*c*) do not sensitize the guinea pig, (*d*) are not precipitating antibodies, (*e*) typically do not neutralize the antigen, and (*f*) are more heat labile. Specific reagins are not found in all allergic individuals, and in some allergic conditions they do not seem to have been demonstrated. (*5*) The term atopy (strange disease) was proposed by Coca to denote certain clinical forms of human hypersensitiveness which are affected by a hereditary predisposition; hay fever and asthma are included, and probably certain other conditions. (*6*) The mechanism of the reactions observed in natural or atopic human allergy is also thought to be basically the combination between antibody and antigen, because: individuals with hay fever, etc., have reagins in their blood; and the reagin content of the blood increases following injection of pollen extracts. The immediate cause of symptoms is believed to be the liberation of histamine from the tissues. The "shock organs" include the conjunctiva, nasal mucosa, bronchi, skin, gastrointestinal mucosa, and nervous system. (*III*) Typical allergic conditions include: (*1*) Hay fever, due to "immunization that does not immunize" by pollen, dust, danders, etc. (*2*) Asthma. (*3*) Atopic dermatitis. (*4*) Urticaria (hives). (*5*) Angioneurotic edema. (*6*) Drug allergy. (*7*) "Familial nonreaginic food allergy." (*8*) Contact dermatitis. (*9*) Possibly migraine. (*10*) Bacterial allergy (Chapter IX). (*11*) Atopic allergy may be treated by elimination of the excitant or excitants from the environment of the patient, "desensitization," and symptomatic treatment (adrenaline, Benadryl, etc.).

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CHAPTER IX

ALLERGY AND IMMUNITY; BACTERIA, VIRUSES, PARASITES

A. Allergy of Infection

The subject we are going to take up now is essentially the same as that called bacterial allergy by some writers, but we shall consider here, in addition, allergy resulting from infections due to certain viruses and parasites. The phenomena are perhaps best understood by the study of specific examples, of which the best known is doubtless the tuberculin type of hypersensitiveness.

1. Allergy in Tuberculosis

Koch (48) made the basic observation that "Tuberculin may be injected into normal guinea pigs in considerable quantities without causing noticeable symptoms. Tuberculous guinea pigs, on the other hand, react to comparatively small doses in a very characteristic manner." Koch found that larger doses would kill tuberculous guinea pigs. He interpreted this as due to the toxic property of tuberculin, the animal's tissues being supposed to be already saturated with as much as they could tolerate of the poison. Work of later investigators has shown that the reaction has a different mechanism; the tissues of the tuberculous animal have become specifically altered by the infection, and tuberculin is entirely harmless to normal animals. It was thought for a long time that true tuberculin sensitization could be produced only by infection with living organisms, but there is some evidence to the contrary. For example, Flahiff (34) has reported producing typical tuberculin sensitivity in children by heat-killed tubercle bacilli. Freund *et al.* (35) report the production of local hypersensitiveness (and antibodies) by injecting heat-killed tubercle bacilli in paraffin oil. See also Olcott (56). Tubercle formation in the animal seems essential in all cases. Tuberculoprotein from tuberculin will sensitize guinea pigs to anaphylactic shock, but will not produce the tuberculin type of sensitivity.

The distinguishing characteristics of the tuberculin type of reaction are: (*a*) there is an incubation period of several hours before the reaction shows itself*; (*b*) the reaction is inflammatory in nature; (*c*) hyperpyrexia is characteristic of the general reaction; (*d*) the symptoms are very similar in different species, and are different from those of anaphylactic shock.

The tuberculin may be injected into the skin, in which case a local reaction is obtained, or subcutaneously; in the latter case a local reaction (at the site of injection) and also a focal reaction (about the tuberculous lesions, wherever they are) are obtained. The reaction is absent in the very early and in the last stages of infection.

Not only is the response of the tuberculous animal different from that of the normal to the (nonliving) tuberculin, but there is also a striking difference in its response to inoculation of living tubercle bacilli (the "Koch phenomenon"). When a normal guinea pig is injected with living tubercle bacilli, a localized nodule appears at the site of inoculation in 10 to 14 days. This nodule increases in size, involves the skin and undergoes necrosis, leaving a persistent ulcer. In the meantime the disease is spreading through the lymphatics, eventually to lead to the animal's death. But, if living tubercle bacilli are injected into a guinea pig with an infection of one week's standing, the response is (*a*) more rapid; superficial inflammation appears in one to two days, followed by necrosis the next day, leaving a flat shallow ulcer which heals rapidly; and (*b*) not followed by spread of the infection from the new site; for example, the regional lymph glands are not affected.

It will be seen that the tuberculin reaction is quite different in character from anaphylaxis; but in tuberculosis both anaphylaxis and allergy of infection can often be observed (see, e.g., 8, 24, 98).

Thus bacterial disease agents seem to act in one or both of two ways, i.e., to lead to the formation of protective antibodies, or to produce in the host hypersensitiveness or allergy to some constituents of the bacterial cell. The last action itself may be of two kinds, depending on whether there is produced an anaphylactic type of sensitiveness, similar in nearly every way to that produced by ordinary nonbacterial antigens, or the tuberculin type of sensitiveness. The former is shown by the production of an immediate reaction by the injection of a "shocking" dose, the latter leads to a

* The immediate type of reaction has been observed occasionally with PPD (see page 123).

delayed inflammatory type of reaction. Thus an animal sensitized with tuberculoprotein will, on injection of filtrate from a culture of tubercle bacilli, die within a few minutes with typical anaphylactic symptoms. An animal infected with tuberculosis can also be killed by injecting the filtrate, but only after some hours, while the symptoms are those of a slowly progressive toxic effect. The response to bacterial agents may be summarized schematically:

(*1*) Production of protective antibodies.
(*2*) Production of allergy.
  (*a*) Anaphylactic allergy.
  (*b*) Tuberculin type allergy (allergy of infection).

The reason for the difference between the tuberculin and other types of hypersensitiveness is not understood. In fact, a number of workers are convinced that bacterial allergy is on the whole not so very different from other forms of hypersensitiveness. The reader may find a good presentation of this point of view in a paper by Bronfenbrenner (13). It is a fact that tuberculin allergy is one of the more extreme examples of bacterial allergy.

An observation which is probably of considerable significance was made by Dienes and Schoenheit (28, 29). They found that reactions indistinguishable from the tuberculin type could be obtained even with ordinary antigens such as egg white or horse serum, if the proper method of sensitization were used. This method consisted in the injection of the antigen *into the lesions* of a tuberculous animal. Freund and McDermott (36) were able to obtain in guinea pigs reactions to horse serum similar to the tuberculin reaction. This was accomplished by injecting horse serum combined with a lanolin-like substance and killed tubercle bacilli. Dienes and Mallory (27) believe that the tuberculin type of hypersensitiveness represents the first stage of every immune response to injected protein. This is also suggested by some observations of Hooker (42), who found that many untreated individuals who gave "delayed" reactions to horse serum on primary test usually gave, after a course of toxin-(horse) antitoxin injections, the immediate wheal type of reaction when tested with horse serum. It may be that the differences between tuberculin reactions and the reaction ordinarily obtained to other antigens are due to some modifying effect of the inflammatory cellular reactions. The same sort of sessile antibodies which are involved in anaphylaxis and atopic hypersensitiveness could play a role here, for the pathologically altered tissues may possibly respond differently to the antibody–antigen reaction.

Some have supposed that the Dienes effect is due to modification of the injected antigen in the tubercular focus, in such a way that it produces the tuberculin type of sensitization. However, Hanks (38), attempting to test this idea, found it possible to obtain the tuberculin type of response to ordinary antigens without having to inject the sensitizing dose directly into a well-developed focus. He did this by inoculating one testicle of a guinea pig with tubercle bacilli, removing the testicle the following day, and injecting the antigen (horse serum) into the opposite testicle. Ten days later an injection of horse serum produced a skin reaction of the delayed tuberculin type. Hanks concluded that the tuberculin type of hypersensitiveness could be produced without the antigen's being modified by the inflammatory tubercular reaction. It seems not improbable that this conclusion is justified, although it has been pointed out (99) that there might have been even in 24 hours a considerable distribution of the tubercle bacilli through the body with the consequent formation of small foci. Whether it may be supposed that these small and undeveloped foci could alter much of the injected antigen is another question. It might seem equally probable that the essential modification is in the mode of response of the tissues themselves.

2. Arthus Phenomenon

It was found by Arthus and Breton (4) that if horse serum, which is harmless to normal rabbits, is repeatedly injected at intervals of several days, eventually the later injections will give rise to a characteristic reaction, involving infiltrations, edema, sterile abscesses, and in severe cases even gangrene. It is not necessary that the earlier injections be made in the same place as the later ones. This reaction is less easily obtained in guinea pigs and some other animals, but it is fairly common in man. It is specific, and evidently due to a sort of hypersensitiveness, in some ways similar to the allergy of infection.

According to Rich and Follis (65), the cells of the tissues at large are not sensitized, but the tissue death which results is due primarily to impairment of nutrition resulting from vascular damage and clogging of the tissue spaces with exudate and hemorrhage. The studies of Cannon and Marshall (18) indicate a definite relation between the degree of sensitivity and the precipitin titer of the serum. They suggest that the Arthus phenomenon is dependent upon the union within the tissues of circulating precipitin and its specific antigen.

3. Differences between Allergy of Infection and Arthus Type of Hypersensitivity

According to Rich (63) allergy of infection differs from hypersensitivity of the Arthus type in the following ways: (*a*) the slow development of the reaction following contact in allergy of infection, contrasted to the prompt appearance of the reaction in the Arthus-sensitized body; (*b*) failure of the smooth muscle of the body to be thrown into spasmodic contraction in the former reaction; and (*c*) allergy of infection is not transferable passively.

4. Shwartzman Phenomenon

Shwartzman has described a curious phenomenon which is now known by his name (79, 80). It has also been studied in detail by other workers (see 81, 93). This phenomenon is connected with a nonspecific increase in sensitivity, and may be mentioned here.

If a rabbit is injected intracutaneously with a small amount of cell-free filtrate from a culture of certain microorganisms, and 24 hours later injected intravenously with the same or some other suitable filtrate, the intravenous injection is followed within a few hours by the development of a hemorrhagic lesion at the site of the endermal injection. The phenomenon is nonspecific, because it is not necessary that filtrates from the same organism be used for the two injections, or that the two organisms used be antigenically related. Cutaneous sensitization seems to be involved, however (3). A variety of filtrates may be used for either injection. However, not all bacteria contain such sensitizing substances. The reaction is not due simply to an inflammation at the site of the first injection, as other kinds of inflammation may fail to sensitize.

5. Relationship of Allergy to Immunity in Tuberculosis

There has been much disagreement concerning the connection between allergy of infection and resistance. It is clear that when a tuberculous guinea pig is killed by the injection of a filtrate which would be harmless for a normal animal, the allergy resulting from the infection has been of disservice to the guinea pig. However, we ought to consider that, by imposing such unnatural conditions, we may artificially have caused a mechanism which would otherwise be beneficial to produce actual harm.

If we look at the Koch phenomenon, which simulates better the conditions of natural infection and reinfection, it is not hard to believe that the reaction to reinfection is indeed a part of an immune mechanism, and by its energy and promptness, shows an increased ability of the animal to deal with the disease agent. In the case of the guinea pig, it is true, although the secondary lesion heals, this does not confer any permanent benefit on the guinea pig, since it would never recover from the primary infection anyway, but this does not really affect the argument. In the first place, the acquisition of a mechanism to remove, by means of a local ulceration, the new dose of infectious agent is clearly a step in the direction of immunity, and it seems possible that in allergy of infection in general the allergic response at a given site may serve to protect the organism as a whole, although at the expense of more or less damage to the local tissues. In the second place, most animals are not so certain to succumb from tubercular infection as is the guinea pig. In the third place, a number of authors, including Zinsser *et al.* (101), have maintained that even guinea pigs, when rendered allergic (these workers injected dead bacilli), resist the progress of tuberculosis infection better, and remain alive longer, than controls. Rich (63), on the other hand, maintains that acquired resistance does not depend upon hypersensitivity. He cites a number of workers who have reported finding a lack of parallelism between the two phenomena. Zuger and Steiner (102) failed to find correlation between skin sensitivity in inoculated guinea pigs and resistance.

6. Relation of Tuberculin Allergy to Antibodies

It has not been found possible to transfer the tuberculin sensitivity to normal animals by injection of serum from infected animals, so that no circulating antibodies responsible for the altered reactivity have been demonstrated. The altered reactivity of the tissues of the tuberculous animal might be ascribed either to an intrinsic alteration in the cells themselves, or to the production of sessile antibodies in or on the cells. Until a method of deciding between these two alternatives is found, the difference will remain largely a verbal one. The problem must be regarded at present as completely unsolved.

Rich (63) has pointed out that there are nevertheless a number of persuasive reasons for believing that allergy of infection depends upon specific antibody. Among the points he mentions are: (*a*) the fact that antibody can not be demonstrated in the circulation might simply mean that a very small amount is sufficient to produce the allergic state; (*b*) the reaction shows a high degree of specificity, making it difficult to account for it on any other basis; (*c*) allergy of infection exhibits the anamnestic reaction (see Chapter II) as does antibody production; and (*d*) the sensitivity can be depressed or abolished by specific desensitization.

It has been reported (14) that in highly sensitized individuals dying of tuberculosis few or no acid-fast bacilli are found in lesions of the lung, except in areas of necrosis separated by an avascular barrier, whereas, in individuals who had nearly lost their skin response, lesions teeming with tubercle bacilli are found. This suggests that sensitivity correlates with at least some of the mechanisms of resistence.

The various antibodies to constituents of the tubercle bacillus in infected animals, which may also be produced by injecting normal animals with killed organisms, unfortunately do not seem to bear any recognizable relation to the tuberculin hypersensitiveness.

7. Role of Antibodies in Immunity to Tuberculosis

The tubercle bacillus has antigenic properties similar to those of other bacteria, and injection of dead bacilli, or infection with living organisms, produces agglutinating, precipitating, complement-fixing, and opsonizing antibodies. Anaphylactic antibodies are often found, and may easily be produced in guinea pigs by injecting tuberculoprotein from filtrates of cultures of the bacillus. It is hard to believe that these antibodies could be without any protective or curative effect, but so far there is no convincing evidence that they can passively confer any increased resistance. Possibly proper methods of study will yet reveal a protective role for these antibodies (63). This whole topic is one of the most challenging problems of present day immunology. Whatever the mechanism may be, some of the recent work (e.g., Potter, 61, Steiner and Zuger, 84, and Corper and Cohn, 23) indicates that inoculation with living avirulent strains of the tubercle bacillus may confer considerable protection.

8. Diagnostic Value of Tuberculin Response

The characteristics of the tuberculin response make it of value in the diagnosis of the disease. There are a number of methods of administration and preparation of derivatives of the tubercle bacillus for this test. The "Old Tuberculin" prepared by filtering an evaporated glycerin bouillon culture of the bacilli is still used; the purified protein derivative (PPD) prepared from filtrates of cultures in synthetic medium by the method of Seibert is more stable and probably more uniform. The intracutaneous test, originally due to Mantoux, is probably the most used. In this method, diluted tuberculin is injected into the skin, and a reading is made after 48 hours. Redness, edema, and in very strong reactions, some necrosis, constitute a positive reaction (see Chapter XI).

A positive reaction indicates that somewhere in the body there is tissue infected with tubercle bacilli. This tissue may be very small in extent, or the lesion may even be completely inactive, and the reaction may still be positive. It has been found that 90% of adults have small healed or inactive areas of infection, so that a positive reaction of moderate intensity is not of much clinical significance except in very young children. A negative reaction is much more significant, since it shows definitely the absence of tuberculosis, except in the cases of patients in the very early stages, or the last stages, of infection, or with acute fulminating types of tuberculosis ("galloping consumption").

9. Allergy of Infection in Other Bacterial Diseases

The tuberculin type of allergy is not confined to tuberculosis. It occurs in glanders, abortus infections, some fungus diseases (trichophytosis), and various other infections, in all of which it has some diagnostic value. Zinsser and Grinnell (100) found that such a skin sensitiveness could be produced in many guinea pigs by injection of large amounts of pneumococci, or with autolyzed pneumococci. The reactions were, if anything, rather more hemorrhagic and necrotic than severe tuberculin reactions.

On the other hand, it must not be thought that the hypersensitiveness resulting from infection is always of the tuberculin type. In a number of diseases, including some bacterial infections, the skin reactions obtained are of the immediate type. Thus Tillett and

Francis (91) demonstrated an immediate reaction of the urticarial type in pneumonia convalescents; this reaction was type specific, and was thus elicited by the capsular polysaccharide. They also observed skin reactions analogous to the tuberculin reaction, which were not type specific, when pneumococcus protein was injected. The immediate type of reaction is found in a number of parasite and fungus infections.

10. Allergy of Infection in Syphilis

There is some evidence that an allergic mechanism may operate in syphilis, but if so it is not nearly so clearly displayed as in tuberculosis, and some workers, such as Rich, Chesney, and Turner (64), deny that it plays any significant role. A number of earlier investigators noted that after infection with syphilis an animal at first responds to reinfection with an accelerated pathological response, although later a highly but not absolutely refractory state appears. Other workers (references in 99) noted that resistance was likely to be less, following an infection which arouses little or no tissue reaction, than after one when the response is violent with rather extensive lesions. The tertiary lesions of syphilis tend to become more and more localized as time goes on, first in one part of the body, then in another, and are likely to be more destructive, as the interval after the infection lengthens. Hinton (40) has called attention to the fact that this suggests a type of allergy growing out of previous sensitization of the tissues by the spirochetes or their products. Sulzberger (87) goes so far as to say that allergy plays the significant role in the basic mechanism of both human and experimental (animal) syphilis. However, others feel that this is not certain and, in particular, doubt the importance of the allergic mechanism in determining resistance to syphilitic reinfection.

11. Antibacterial Immunity

Having now discussed successively phagocytosis, antibodies, and bacterial allergy, we have considered the main factors involved in antibacterial immunity. It would be difficult, and probably pointless, to attempt to assess the relative importance to the host of these different factors. Antibodies have a more or less unique position, since they have direct protective action, and also their presence is necessary, or at any rate extremely helpful, for at least one of the other phenomena, phagocytosis.

12. Allergy of Infection in Virus Diseases: Smallpox

The altered reactivity of the skin demonstrated by the changed course of cutaneous reinoculations with vaccinia virus is probably an example of allergy of infection. In an individual who has never had smallpox and has never been vaccinated, the first inoculation with virus usually causes a reaction which appears after an incubation period of about three days and reaches its maximum between the eighth and eleventh days. In a previously exposed individual with high immunity, the reaction is accelerated, being condensed into a period of a few days. By the seventh day the site is already entirely healed. In individuals with less immunity, the reaction may take longer, and response will be intermediate in character (see Chapter XI). These types of reaction are characterized as vaccinia, reaction of immunity, and vaccinoid, respectively.

Hooker (43) showed that the hypersensitiveness of infection following smallpox vaccination could be demonstrated by injection of dead virus into the skin. This both confirms the idea that the early "reaction of immunity" is allergic in nature, and affords a method of detecting immunity without the use of living virus, for it was found that the degree of the reaction obtained correlated well with the degree of immunity as demonstrated by later response to inoculation of the living virus.

B. Virus Diseases

1. Viruses

The diseases caused by viruses seem to have certain characteristics in common, such as to justify our treating them as a group. According to Rivers (66) these characteristics are frequent presence of inclusion bodies in affected cells, appearance of inflammation mainly as a secondary phenomenon, and proliferation and/or degeneration as the primary cellular changes.

At least two virus diseases, Shope's papilloma (78) and Rous's sarcoma (70) are difficult to distinguish from tumors, though some workers prefer not to speak of them as tumors. There is evidence (37, 46, 55) that acquired tumor immunity is at least in some cases immunological in nature, and antibodies have been observed (46, 55) to be somewhat effective in certain cases. The question arises whether many or perhaps all malignant growths are also caused by viruses. It seems to some workers that the trend of much recent work on the tumor problem is to indicate that this may be so, but a final proof will await the demonstration of the active agent in such growths, which has not yet been accomplished. Many authorities, however (see 66), treat this suggestion with skepticism.

2. Immunity in Virus Diseases

Just as in other diseases, susceptibility to viruses seems to be dependent on a number of factors other than immunity acquired as the result of exposure to the virus. Some viruses are so specific that they attack, so far as we know, only one species (e.g., infectious papilloma of Shope and salivary gland disease of guinea pigs).

In most virus diseases lasting immunity follows an attack. With some, however, repeated attacks occur. Some believe that in such cases virus remains in the body of the apparently fully recovered animal, as has been found to be the case with some virus plant diseases. If the living virus does so persist, it might be possible that in some cases, as when the host is weakened by other agencies, his immunity might be lowered to the point where the virus could again grow rapidly and produce a new infection or a relapse. This explanation has been advanced for the recurrence of fever blisters (14a), and it is one of the possibilities in the interesting case of the common cold.

3. Mechanism of Antivirus Immunity

We have seen above that two factors might be involved in active immunity. The cells of the animal's tissues might perhaps develop an intrinsic resistance, or circulating antibodies may be produced. There are those who believe that in antivirus immunity the former is the important thing, and that the role of the circulating antibodies is a minor one. This is supported by the observation that animals may apparently have some antibodies producing agglutination, precipitation, and complement fixation, without being immune or resistant to vaccinia infection (60). Also, in some diseases, such as lymphocytic choriomeningitis in mice (94), recovered animals are refractory to reinfection, but no neutralizing antibodies have been demonstrated. Webster (95) and Hodes and Webster (41) showed that mice vaccinated with St. Louis encephalitis virus are highly immune at the end of six weeks, though few or no neutralizing antibodies are yet demonstrable in their serum. The neutralizing antibodies reach their maximum 22 to 24 weeks after vaccination, when the animals have already lost their resistance to infection. Human beings with what was considered to be an adequate amount of neutralizing antibody have nevertheless been observed in some cases to contract poliomyelitis (12).

Other workers, particularly Bedson (9), believe that the antibodies play the important role. Salaman (74) found that tissue cultures of cornea of rabbits immune to vaccinia were just as susceptible as normal cultures, suggesting that no alteration of the nonvascular tissue, at any rate not one capable of being propagated, was responsible for the immunity. It has been pointed out that failure in any given case to find antibodies does not prove they are not there, as the amounts might be too small to be detected by present technic; also the possibility that antibodies may be present, but attached to the cell ("sessile") must be considered. It would seem that a final answer to this question must await further work.

Antivirus antibodies capable of protecting against measles and infectious hepatitis have been found in the γ globulin separated from plasma (58, 85, 86). Circulating antibody for mumps virus has been demonstrated by Enders (31). In most cities the serum of the great majority of adults contains antibodies capable of neutralizing considerable amounts of influenza virus A (15).

Antivirus antibodies in an animal's circulation may be tested for by agglutination, precipitation, complement fixation, or neutralizing power. All of these forms of antivirus activity have been observed. In the case of some viruses it is probable that some of these antibodies, at any rate, are not identical. There is evidence that the neutralizing antibodies are different from the others. In the case of vaccinia, it has been found that complement-fixing antibodies, agglutinins, and precipitins can be obtained by injecting the purified substance S (59), or completely inactivated elementary bodies (60), although practically no neutralizing antibodies are formed. The neutralizing antibodies are absorbed by washed elementary bodies, but not by the soluble specific substance (74).

It was at first thought that the neutralizing antibodies killed the virus. It has been shown by Todd (92) and Andrewes (1) and others that neutral mixtures can be made active again in some cases by simple dilution or centrifugation. Such observations were at first thought to show that no combination took place between the neutralizing antibodies and the virus, but Andrewes (2) and Salaman

(73) later showed that the mixtures did not become active on the above treatment if first allowed to stand for sufficient periods of time. Salaman (74) points out that the fact that neutralizing antibody can be absorbed by elementary bodies is evidence for real union.

The interesting observation has been made that the neutrality or lack of neutrality of a mixture of virus and serum depends to some extent upon the organ into which it is injected for test. Certain virus–serum mixtures have been found to be neutral when injected into the skin, but still somewhat active when injected into the brain (1, 72) or the testicles. It has also been found (see 99) that, when vaccinia cultures are made with normal serum, the virus attaches itself to the tissues promptly and multiplies. In cultures made with immune serum, the virus similarly becomes attached to the tissues but does not multiply. Such tissue–virus mixtures can be washed until no neutralizing antibodies are found in the washings, but the tissue will still not become infected, though it is infectious for other unprotected tissues. Extended washing will render even protected tissue susceptible. Salaman (74) with Robinow found that in tissue cultures of rabbit's cornea, if virus is applied first and then antiserum, infection is not prevented. They also found that, when elementary bodies are mixed with antiserum, centrifuged, washed, and resuspended, their infectivity is much reduced. Salaman thinks the most probable explanation of these results is that the antiserum may act in two ways to prevent entry of virus into the cell: by attaching itself to the virus, or to the host cell surface, or to both. Once virus has reached the interior of the cell, antiserum can do nothing to prevent its survival and multiplication there.

If Salaman's explanation is correct, we might speculate that the reason different organs may indicate different degrees of neutralization for the same mixture of serum and virus may be that the antibodies attach themselves more readily to some tissues than to others, or perhaps that to prevent its entry into certain of the more permeable tissues it is necessary to have more antibody combined with the virus. Or both of these factors might play a role.

The above discussion has been illustrated chiefly by results obtained with vaccinia. Results with other viruses are fewer, but on the whole similar, although each virus presents its own problems and peculiarities (67). We can not attempt to summarize here the studies on other viruses.

C. Immunity to Parasites

Animal parasites as well as bacteria and viruses of course contain antigenic substances (see Chapter IV), and it is to be expected that immunization, in the broad sense of the word, will occur whenever these antigens reach the circulation of the host. The result is sometimes a hypersensitivity of infection, and the disease can often be diagnosed in such cases by skin tests, as in the case of infection with echinococcus or trichina.

Circulating antibodies may also be produced, and may be detected by precipitation, or by complement fixation. In the case of worms living in the alimentary canal such tests seem to be less reliable, perhaps because less unchanged protein or other antigen from the parasite ever reaches the host's circulation, so that the degree of immunization remains less.

There is no doubt that acquired resistance plays an important role in protozoan and metazoan infections, just as in bacterial infection. It is well known that protozoan infections, after an acute attack, usually go into a chronic state in which it will be found that the parasites are few and difficult to demonstrate. This state may be interrupted by relapses. During the chronic state there is usually marked resistance to reinfection. Thus adults native to highly malarial countries do not develop acute malaria, and chronic carriers of amoebic dysentery do not seem to develop amoebic dysentery.

It is not always easy to demonstrate antibodies in such cases, partly because of technical difficulties, but Coggeshall and Kumm (21) demonstrated that the serum of rhesus monkeys with *Plasmodium knowlesi* malaria conferred a passive immunity on normal monkeys, and Coggeshall (20) found protective and complement-fixing antibodies were produced in human beings following experimental infection with *Plasmodium knowlesi*.

In the case of worms the increased resistance seems to take two forms: interference with maturing of the parasites after entry, and resistance to injurious effects of the parasite. Natural immunity appears to depend partly on differences in the action of the gastric juices; there is evidence that antibody production is concerned in acquired immunity.

It has been definitely demonstrated that rats infected with *Trichinella spiralis* develop a considerable immunity to reinfection (7, 30, 51). The immunity was thought to be localized largely in the intestine. McCoy (52) obtained various degrees of artificial immunization by repeated intraperitoneal injections of living or dead trichina larvae. The resistance of the artificially immunized animals did not affect the initial development of the adult worms in the intestine, but caused their rapid elimination. In the naturally infected animals both effects were observed. Sarles (75) was even able to observe, following the administration of immune serum, the expulsion of worms already established in the intestine.

Taliaferro (89) believes that the chief mechanism of immunity in such cases is the formation of specific antibodies. These may have three main effects: they may form a specific precipitate around the parasite, thus immobilizing it, and perhaps also stunting its growth; there may be specific antibodies formed for enzymes of the worm which are instrumental in digesting and attacking host proteins; and the antibodies may have some part in increasing cellular response, leading to walling off of the immobilized parasite and mobilization of macrophages to remove the dead parasite.

Wright (97) found in rabbits injected with *Trichinella spiralis* that, as immunity developed, there was an increase in the proportion of γ globulin in the serum.

Taliaferro and Sarles (90) have described the process of resistance of the skin of immune rats to invasion by larvae of *Nippostrongylus muris.* After the larvae penetrate the skin they become somewhat stunted, coiled, and immobilized. Precipitates are observed to be formed in and around them. These precipitates are considered to be the result of combination of antibodies produced by the host and antigenic constituents of the worm. In addition to immobilizing and sometimes killing the worms, they increase the intensity of the inflammatory response. A few larvae are retained in the skin and occasionally one dies. Concomitantly, inflammation, more intense and involving more hematogenous cells than in the non-immune rat, develops, proceeding occasionally to the formation of large diffuse nodules around the larvae. The worms however remain alive for long periods in such nodules and many escape. Those that do not escape eventually die, and the macrophages in the nodule remove the debris. In higher degrees of immunity more worms are retained in nodules and die.

The nodules are formed by the migration of leucocytes from the blood vessels, and their accumulation around an immobilized worm.

Edema and hemorrhage are often present. The fully formed nodule consists of a coiled, immobilized worm containing precipitates and surrounded by closely packed cells, including macrophages, fibroblasts, etc.

Passive transfer of immunity to parasitic infection has been demonstrated (19, 75, 76), and all the manifestations of active immunity, with the possible exception of the death of the worms, can thus be duplicated.

There is probably a considerable diversity of antigens in a single worm, and Canning (17) found comparatively sharp tissue specificity in *Ascaris.* Oliver-González (57) has also made observations on the parts of this parasite to which antibody is directed. Probably not all of the antigens are concerned in the actual development of resistance. Campbell (16) obtained a purified polysaccharide antigen from the larval form of *Taenia taeniaeformis* which stimulated the formation of precipitins, but did not immunize against infection, and did not absorb the protective antibody from immune serum. Hassid *et al.* obtained an immunologically active polysaccharide from *Coccidioides.* It gave a positive skin reaction in sensitized individuals and also gave positive precipitin reactions.

The reader who desires further information will find the subject of immunity against animal parasites reviewed by Taliaferro (88, 89) and by Culbertson (25, 26).

SUMMARY

(*A*) *Allergy of Infection* (often called bacterial allergy). We take tuberculosis as the paradigm. (*1*) Koch observed that tuberculin injected into normal guinea pigs causes no symptoms, but gives characteristic reactions in tuberculous guinea pigs. The distinguishing characteristics of the tuberculin type of reaction are: (*a*) several hours elapse before the reaction; (*b*) the reaction is inflammatory; (*c*) hyperpyrexia is characteristic if the reaction is general; (*d*) the symptoms, similar in different species, are different from those of anaphylaxis. The possible responses of the body to bacterial agents may be summarized as: (*a*) production of protective antibodies, and (*b*) production of allergy (α, anaphylactic allergy, and β, tuberculin type allergy). The reason for the difference between α and β is not clear; some suppose that β is the first to develop and that it may or may not turn into α. (*2*) The Arthus phenomenon consists in local reaction or necrosis at the site of an injection after a subsequent injection. (*3*) Allergy of infection and the Arthus type of sensitivity differ, since (*a*) the former reaction develops slowly, (*b*) the former state does not sensitize smooth muscle, and (*c*) the former sensitivity is not transferable passively. (*4*) The Shwartzman phenomenon is a nonspecific increase in sensitivity manifested by a hemorrhagic lesion at the later injection site. (*5*) Although many do not agree, it seems possible that the tuberculin type of sensitization may be in some cases, such as tuberculosis, a step in the direction of immunity. (*6*) Although tuberculin sensitivity can not be transferred passively, there are reasons to believe that it may depend on antibodies. (*7*) Antibodies are produced to the tubercle bacillus, and vaccination with living avirulent strains may confer considerable protection, but the relation between the known antibodies and the protective mechanism is still obscure. (*8*) The tuberculin reaction has diagnostic value, since it demonstrates previous sensitization to the tubercle bacillus. (*9*) Allergy of infection may occur in other diseases, such as glanders, abortus infections, and some fungus and parasitic diseases. Sometimes the reactions obtained (e.g., Tillett and Francis reaction in pneumonia) are immediate. (*10*) There is some evidence that an allergic mechanism may operate in syphilis, but there is not general agreement about this. (*11*) Considering antibacterial immunity as a whole, we may say that nonspecific factors, phagocytosis, antibodies, and bacterial allergy all play a role. Antibodies may be the most important in many cases. (*12*) In virus diseases (paradigm, smallpox), allergy of infection is observed. This is one of the factors in the reaction of immunity obtained on vaccination of immune individuals. (*B*) *Virus Diseases.* (*1*) The diseases caused by viruses may have certain characteristics in common (page 358). (*2*) Immunity to viruses, as to bacteria, depends upon a number of factors. Lasting immunity may follow one attack. Sometimes the virus (e.g., herpes simplex) persists for long periods in the body, with or without manifestation. (*3*) There is not general agreement as to whether or not antibodies are primarily responsible for antivirus immunity. Antibodies are at any rate sometimes found. (*C*) *Immunity to Parasites.* Parasites, as well as bacteria and viruses, contain antigens, and antibodies to these are frequently developed by the host, and have been shown in some cases to be important. The demonstration of such antibodies is not always easy. Skin sensitization is also often observed.

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CHAPTER X

PRACTICAL USE OF IMMUNITY, ARTIFICIAL, AND NATURALLY ACQUIRED

1. Preliminary

We stated in this book at the outset that immunology, being a practical science, was directly concerned with a study of the immune mechanisms by which the higher organisms defend themselves against infection, and with methods of increasing artificially the resistance of such organisms to infections they are likely to encounter. Having studied the immune mechanisms in some detail, we may now turn to the question of their practical application.

It may have been the hope of early immunologists that knowledge experimentally acquired would eventually enable us to protect against, or cure, all the diseases afflicting mankind. If so, we are forced to admit that their hopes have by no means been completely realized as yet. We are able to confer on most susceptible persons fairly good immunity to a number of diseases, such as smallpox, diphtheria, tetanus, scarlet fever, and whooping cough, and we are in the possession of sera which have considerable curative power for other maladies, such as pneumonia and diphtheria. Nevertheless, there remain many dread diseases, such as tuberculosis and syphilis, where the immunologist is helpless, or the value of his ministrations is in doubt. We may pause to ask ourselves why this should be.

2. General

Immunological procedures have found important application in prevention, diagnosis, treatment, and testing for immunity in a number of diseases. It is not easy to say why immunological procedures have been so much more successful in preventing, treating, or diagnosing some diseases than they have with others. The body probably makes some immunological response in its defense effort against any infectious agent. Since in some cases protection of higher or lower degree can be produced by the injection of a dead or attentuated agent, the question may be asked, why can this not be done in all cases? The full answer to this question must await further investigation, but experience suggests certain ideas which may be applicable. For example, some diseases seem intrinsically to produce less immunity in the host than do others. It is understandable that immunization, which probably seldom produces an immunity as lasting and solid as that following a natural attack, should be relatively ineffective in such cases. Adequately controlled experiments, made during the recent war, on vaccination against malaria indicated that this procedure, as carried out, was unsuccessful (40).

Sometimes, evidently, the same disease (to all appearances) may be caused by different infectious agents, or by different strains of the same agent. Immunity to one of these agents or strains does not necessarily imply any high degree of immunity to another, and attempts at artificial immunization may fail to be fully successful because only one or a few of the antigenic types are represented in the vaccine.

Some individuals seem congenitally incapable of acquiring a high degree of immunity to certain diseases, while others rapidly build up a high degree of resistance. It might well be thought that with diseases showing such variation artificial methods of immunization would be of little use, since the naturally susceptible individual who cannot be made immune by an attack of the disease will hardly be expected to benefit much from the generally less potent stimulus of artificial immunization.

In many diseases, of course, the immunological procedure is obviously of minor importance, compared to other methods such as clinical diagnosis and chemotherapy (especially when the disease is due to a microorganism susceptible to some available antibiotic).

Immunization is probably most successful in diseases in which antibodies play a relatively important role in natural defense. In diseases in which cellular reactions are more important, injection of dead organisms tends to confer less protection. This may possibly have a bearing on our failure to vaccinate very successfully against tuberculosis. Another factor is the antigenic constitution of the infectious organism, as it exists in the body, and as we cultivate it *in vitro*. Often a significant difference has been found between the two forms, and the cultivated organism lacks important or essential antigens for the production of good immunity. This may be partly responsible for our failure to immunize artificially against syphilis. Another important factor is that we are often limited as to the amount of material we can introduce during immunization. With virus diseases, particularly, this may be an essential factor in our failure to obtain good immunity artificially (see Chapter IX). Some microorganisms seem to be intrinsically better stimuli to the formation of antibodies than are others, in analogy with what we have observed of antigens in general (see Chapter III). The immunizing power of an antigen depends upon its possessing a particular chemical configuration, not upon the virulence of the organism in which it occurs (23). Finally, in a number of cases our success has depended upon our being able, by hook or crook, to obtain a modified infectious agent which still has the power to invade the body and produce good immunity, but does not possess the power to produce dangerous disease. This is particularly true in virus diseases.

Modification by passage through individuals of other species is frequently used (e.g., 74) to produce such an "attenuated" agent, and may have been the mechanism by which vaccinia virus arose from smallpox virus (23).

No one method of preparing immunizing agents can be expected to be applicable in general. The method will vary with each organism and even with the particular antigen of the organism which it is desired to preserve. The right immunizing agent can only be found by observation and experience (23).

It should also be kept in mind that it may be a positive disadvantage to sensitize, or produce antibodies to an antigen, if the antibodies produced have no protective power. It is desirable to produce, when possible, only those antibodies to an infectious agent which aid the body in resisting it.

The use of adjuvants, such as oils from petroleum, and lanolin-like substances, to enhance the immunizing power of vaccines and toxins is becoming more common (30–32). See page 84.

The extent to which immunizing procedures, even if successful, will be applied practically, will depend on the prevalence and severity of the disease in the locality in question. If the disease is too rare, or seldom dangerous, it will not pay to undertake mass immunization.

Not all immunologists, probably, would concur in this last statement. Some might consider it desirable to immunize routinely, should it eventually prove possible, to certain diseases which are not highly dangerous, such as measles, mumps, and the common cold. Considering the loss in working hours and efficiency which annually results from this last disease, we must admit that a satisfactory method of mass immunization might be of sufficient importance to justify our undertaking it on a national scale. Immunologists in general agree, however, that no procedure thus far tried for producing immunity to colds has proved to have any decided effectiveness (54).

3. Production of Antibacterial Immunity

Before we consider specifically artifically induced immunity to bacterial diseases, we may do well to pause and remind ourselves of the natural course of a bacterial infection, when the body is left to deal with the invaders with its own resources. This topic was discussed in Chapter I (page 23).

From our discussion it will be seen that during an attack of a bacterial disease the body comes into intimate contact with large numbers of living micro-organisms, and doubtless has the opportunity of manufacturing antibodies against a number of the antigens contained in these bacteria. For the artificial production of immunity we shall rarely wish to take the risk of administering living virulent organisms to our patients. It would seem that the next best procedure, from the point of view of the production of an effective antigenic stimulus, consists in the injection either of killed, washed cultures (called vaccines) of freshly isolated strains of known virulence, or, if the harmful effects of the organism in the human body are due mainly or entirely to the action of a soluble exotoxin, injection of the latter substance, or some nontoxic but antigenic modification of it. In some cases, as possibly with *Hemophilus pertussis*, even washing seems to risk removal of constituents of the organisms important for antigenicity.

We are by no means always able to keep to the above prescription in artificial immunization to all bacterial diseases. In a number of cases we are forced, for one reason or another, to make use of preparations which almost certainly lack an important antigen. A good example is the Vi antigen of the typhoid bacillus, which is so unstable that it is damaged or destroyed by killing the organisms. Since, however, we could not venture to use live typhoid bacilli for our vaccine, we were obliged to use killed, less potent, preparations. In spite of this, the results obtained have proved well worth while (page 450). Details may be found in (15, 78). Methods of preparing and preserving killed typhoid vaccines still retaining considerable Vi antigenic activity have been reported more recently (1, 67).

In other cases, too, there is evidence that injection of killed organisms has distinct prophylactic value. In pneumonia, there is some evidence that vaccination with killed organisms, or with specific carbohydrate from the organisms, will confer some degree of immunity to the particular type of pneumococci used (17, 28, 80, 96). It seems, however, that this immunity is of short duration, probably not more than a few months (53, 85). Due to this fact, and to the large number of types of pneumococci, mass immunization of the general population hardly seems practical, but immunization of soldiers, sailors, inmates of institutions, and others during pneumonia epidemics, may be utilized probably with profit. Heidelberger (40) was able to immunize to a number of pneumococcus types (using the specific polysaccharides) so as to break up epidemics in the armed forces of the U.S.A. during the late war (40a). Sherwood and Heathman (77) obtained "very high humoral immunity" by injecting vaccines of killed amoebae.

Tuberculosis provides us with an example of an important disease where thus far the serologist has been able to accomplish rather little (page 355). Vaccination with a living avirulent strain of the tubercle bacillus, called B.C.G. (Bacille Calmette-Guérin), was introduced by Calmette (16). It has been extensively tested, particularly in French-speaking countries, but English-speaking writers until recently on the whole were unimpressed with its value and safety (e.g., 38, 90, 96). There are of course some exceptions to this statement, for example, the paper of Aronson (5). More recent work with B.C.G. and other living avirulent vaccines in this country has suggested that they may be of definite value (22, 64, 84).

In attempting to immunize against some diseases, the high toxicity of the killed organisms presents a difficulty, and attempts must be made to isolate some antigenic component, of less toxicity, from the cell, or to detoxify a toxic component.

A number of fairly successful attempts to accomplish this sort of thing have been reported. Goebel *et al.* (36, 63) reported that inoculation with the purified specific antigen of type V *Shigella paradysenteriae* (Flexner) produced agglutinins and mouse-protective antibodies in man. Morgan and Schütze (57) obtained similar results using the O antigen of the Shiga bacillus. Day (21) found that a species antigen from pneumococci, carefully separated from an "opposition factor," would immunize mice. Dubos (22) has pointed out that in general it is the surface antigens which are needed for effective immunization, but that not all surface antigens will work. Treffers (92) was able to detoxify soluble antigens from *Shigella dysenteriae* (Shiga) and *Eberthella typhosa*. Evans (25) used formaldehyde to detoxify a toxic extract of *H. pertussis*, obtaining an antigenic product. Smolens and Mudd (81) isolated a fraction of *H. pertussis* which was thought to be useful in skin tests for susceptibility to whooping cough and possibly of some value in reinforcing waning immunity.

Even a synthetic antigen, if it possessed the proper specificity, should produce the desired immunity, and an approach to this has been made by Goebel (33–35). See page 113.

4. Antitoxic Immunity

When a toxin produced by the invading organism plays the chief role in its harmful effects, we may expect in most cases to be able to immunize with toxin.

Active immunization against diphtheria has been found to be very effective in preventing the disease. Numerous studies have established the value of the procedure beyond question (4).

Immunization with diphtheria toxin directly is not very practical because of its high toxicity. The use of toxin–antitoxin mixtures has been abandoned in favor of toxoid. The nature and mode of preparation of toxoid (toxin detoxified by formalin) are discussed on page 407. It has been found as effective as toxin–antitoxin as an immunizing agent. The technic of immunization is described in the following chapter.

The immunity conferred by diphtheria immunization is not absolute, as a few cases of diphtheria in immunized children have been observed. It seems that in such cases the disease follows a milder course, however, so not all benefit has been lost. But there is abundant statistical evidence that the majority (about 90%) are protected. Tests indicate that after four or five years a not inconsiderable proportion of children may require reinforcement of their immunity (which can be accomplished by a single dose). This is particularly necessary if the immunization were done before the age of one year, as is to be recommended.

Immunization with scarlatinal toxin will make most individuals Dick-negative (page 474). Whether this does more than protect against the rash is still disputed (48). Toomey (88) thinks it does.

In any case, the rash, beng a toxic manifestation, is perhaps an indicator of more generalized damage done by the disease, which is one that can be followed by serious *sequelae*.

It has been demonstrated by a number of workers that immunization with tetanus toxoid (formalin-detoxified tetanus toxin) will produce in human beings a good titer of antibodies, and in all probability considerable immunity to the disease. See references in Ramon (68) and Janeway (44). The period between injections should be fairly long (six to eight weeks), and it seems advisable to give a "reminder" dose nine months after the first dose. Hall (37) reported that alum-precipitated toxoid seemed superior to plain toxoid. The immunity seems to last for a considerable period of time (one to four years), and can be easily restored by a later single injection, apparently even after a wound or injury has been received which might result in tetanus. Mueller *et al.* (58) have shown that satisfactory antitoxin response is obtained by the injection of tetanus toxoid made from toxin from bacteria grown on a purely synthetic medium. This toxoid gives fewer undesirable reactions than the older preparations, and the routine use of it or some similar preparation seems becoming widespread.

Good immunity seems to be obtained by the injection of alum-precipitated gas gangrene (*Perfringens*) toxoids (51).

5. Antivirus Immunity

It is an old observation that the immunity resulting from many virus diseases is a lasting one, in many cases good for life. This holds as a general rule in spite of some notable exceptions, such as the recurrence of fever blisters, and the very short period of immunity following an attack of the common cold. Three possibilities present themselves to explain the persistence of virus immunity; (*a*) such viruses may be very potent antigens, so that the body forever after retains some antibodies, or the ability to make them on short notice; or viruses, being present inside the cells during infection, may thereby afford a more powerful antigenic stimulus; (*b*) the immunity resulting from the infection may be reinforced at intervals, or almost continuously, by new contacts with the active agent; and (*c*) the virus may persist in the apparently fully-recovered host, thus providing a continual antigenic stimulus.

The second of these possibilities would hardly explain the persistence of immunity, and of humoral antibodies, for as long in some cases as

50 or 75 years, for example (75), in individuals having once had yellow fever, and having resided subsequently in areas where the disease is not found. The trend of evidence today seems to indicate that (*c*) is the more probable, at least in some cases. The causative agent has been recovered from the immune host in the case of certain plant diseases due to viruses (50), and from animals in the case of various infections, including infectious anemia of horses (49), salivary gland disease of guinea pigs (19), psittacosis (9, 55), and lymphocytic choriomeningitis of mice (91). Burnet (13) believes the virus of herpes simplex persists in the human body for a long time after initial infection, perhaps for life.

It would certainly seem easy, *a priori*, for viruses to persist in a host, since they are located inside the cells, and providing they do not by their presence kill the host cells, they might multiply and pass into the new host cells as cellular division takes place, without being acted on by circulating antibodies, which are outside the cell (70, 73). This seems to happen in a number of virus diseases, such as in the virus tumors of chickens and rabbits. The presence of the living virus in the recovered host would not necessarily mean that he was capable of infecting others and spreading the disease, as the virus might be restricted to parts of the body not communicating with the outside.

6. Immunization to Viruses

We may obtain active immunity simply by provoking an attack not distinguishable from the natural disease, or by producing an atypical reaction by the introduction of the fully active virus through unnatural portals of entry. The old practice of variolation is an example of the latter. Here virulent smallpox virus was scratched directly into the skin, instead of being allowed to enter the body through its natural route, the upper respiratory tract. This was claimed to produce a comparatively mild disease, recovery from which gave immunity. There was a similar old practice of controlling sheep pox (see 43). Rivers utilized this principle in vaccinating for psittacosis in the laboratory (69).

The use of fully virulent virus for the production of immunity in human beings is not, and is not likely to become, popular. Aside from the possibility, always present, that a typical, perhaps fatal, form of the disease *might* develop, there is the danger that the inoculated individual may be infectious to others, and might even start an epidemic.

Therefore, even if routes of inoculation (of virulent virus) which would not produce serious illness were known for all viruses attacking man, it would still be highly desirable to find other methods of producing immunity, if such were possible. This led historically to attempts to produce immunity by use of an "attenuated" virus. The classical example is provided by Jenner's (47) use of the virus of cowpox to produce a mild infection which protects against the much more severe smallpox. Pasteur, working with chicken cholera, first perceived the mechanism of the Jennerian method of vaccination against smallpox and realized that the same principle could be used to establish resistance to bacterial infections (23). Pasteur later applied the principle to the artificial production of an "attenuated" rabies virus (62). By an "attenuated" virus was evidently meant one which had lost all or part of its disease-producing power, but which antigenically was still essentially the same thing. At that time workers apparently did not ask themselves what such a change signified in terms of chemical structure, and even today the point is not thoroughly understood. After 150 years, Jenner's cowpox vaccine remains the most reliable and perhaps the safest of all the methods of immunization to viruses.

Since immunity to various bacterial diseases can be produced by the injection of killed organisms, it was natural to try immunization against virus diseases by injection of killed or inactivated virus. Work in this connection has not been at all uniformly successful. As Rivers (69) pointed out, it is very difficult at present to determine when a virus has been completely inactivated. Ross and Stanley (72) were able partially to reactivate tobacco mosaic virus which had been inactivated by formalin. Kelser, in Zinsser's laboratory, recovered *virus fixé* from the brain of a dog paralyzed after an injection of supposedly killed rabies virus. These facts illustrate how difficult it is to be sure an "inactive" preparation of virus which immunizes is really "dead." In many cases the immunity produced seems to be slight and transitory. In the cases where immunization has apparently been achieved, some have doubted if the virus were really inactivated. In other cases (9), the inactivated virus seems to protect against outward evidence of the disease, but not against infection, and virus can be obtained from the vaccinated and experimentally inoculated animals. Several workers (10, 94), however, found that the injection of sufficient amounts of inactivated vaccine virus would produce an immune state equal to that resulting from infection. The amounts needed were of the order of milligrams, but Weil and Gall

(94) pointed out that they are perhaps no greater than the amounts of living virus produced in an animal during the course of the disease itself.

Mackenzie (52) reported that with large doses of inactive Rift Valley fever virus, administered intra-abdominally, he was able to establish a good resistance to the infection. Zinsser *et al.* (96) found that, although it was formerly always supposed that immunity to typhus could be produced only with the living organism, the injection of killed *Rickettsiae* in amounts quantitatively comparable to those injected in typhoid vaccination would produce immunity. Smadel and Wall (79) succeeded in immunizing guinea pigs with suspensions of the formolized, washed virus of lymphocytic choriomeningitis. It might be assumed that the chief reason inoculation with killed virus does not always immunize is that insufficient amounts of the virus substance itself are used, since such preparations usually contain only a small fraction by weight of virus. Also, in inoculation with the active virus, the introduced virus proliferates and thus ultimately a much larger amount of virus substance is available to act as an antigenic stimulus. This clearly puts any method using small amounts of inactive (dead) virus at a disadvantage.

The classical instance of antivirus immunization is smallpox vaccination. This is described in detail in the following chapter. Next comes, historically, antirabies immunization.

The incidence of human rabies is not high enough to justify mass immunization, but, since the incubation period of the disease is long (40 to 60 days), we are fortunately in a position to immunize only the persons who need it, that is, individuals who have been bitten by an animal known or suspected to be rabid. In case of doubt, it is probably best to immunize a patient who has been bitten, in spite of the rather severe reactions which sometimes follow, since the disease, once it develops, is believed to be always fatal. According to Cornwall (20), about 35% of persons bitten by rabid dogs would, unless treated, develop the disease.

The original Pasteur method, still in use to some extent, involved the injection of suspensions of the central nervous systems of animals dying of the "fixed" virus, starting with material which had been attenuated (dried over sodium hydroxide) for two weeks, and following this each day with less and less attenuated preparations. Later it was found unnecessary to dry the first preparation for so long. A newer method, that of Semple (76), seems to be displacing the original method in many places. It employs a virus preparation which has been treated with 1% phenol for about 24 hours. The final product is a 4% suspension in 0.5% phenol. Daily injections of about 2.5 cc. are given each day for 15 days. More recently a vaccine inactivated by irradiation with ultraviolet light has been suggested (93), and this treatment has also been suggested for other viruses (56).

Formalin-killed influenza virus seems to have proved effective as a vaccinating agent (41, 42, 83), although the duration of immunity is limited, and seems to be restricted to the strain of virus used, as it is following recovery from the actual disease. For a general discussion of immunity to influenza, see Burnet and Clark (14). Toomey (8, 9) has discussed the production of active immunity to smallpox, diphtheria, whooping cough, tetanus, and typhoid in an excellent review article.

7. Duration of Artificial Active Immunity

We may probably state as a general rule that artificial active immunization does not give protection for as long a time as does a natural attack of the disease. (It has however been claimed by some that smallpox vaccination is more effective in preventing subsequent attacks of smallpox than is an attack of the disease itself.) When immunization has been accomplished by the injection of dead organisms, or nonliving products of the organisms, as is usually the case, this is not very suprising. The amount of material we have introduced is probably very much less than that which the body has to deal with during a natural attack of the disease, and it is introduced suddenly, at intervals, instead of gradually over a period of time, and thus may be thought not to offer as good a stimulus to the immune mechanism of the host. In addition, there may be antigenic differences between our artificial antigen, and the actual infecting organism.

We have seen that the host may produce antibodies to a number of different constituents of the bacterial cell. Not all of these antibodies have equal protective power. Thus, in pneumonia it is observed that the antibody directed against the nucleoprotein has little, if any, protective effect (6, 7); it is the type-specific anticarbohydrate antibodies which are important. Of course we can not infer from this that no other antibody plays any role in protection against pneumococci, but it has been established that the anticarbohydrate antibody alone is effective (see page 113).

In the case of the motile bacilli, the results of modern experimental studies have shown that antibodies to the flagellar antigens play little if any role in specific antibacterial immunity; it is the antibodies to the heat-stable somatic antigens, and possibly certain labile antigens important for virulence, which are all-important (26, 27).

Even when we take account of this, however, there are some puzzling differences in the duration of artificially induced immunity. It seems likely that immunity to diphtheria, induced by injecting enough toxoid to make the Schick reaction negative, lasts in most cases for the rest of the life of the individual. Artificial immunity to typhoid or to pertussis clearly lasts a shorter time in many cases, just how long is not precisely known, but perhaps for three to five years in the case of typhoid. Artificial immunity to pneumonia seems to last for only a few months. The greater duration of artificial immunity to diphtheria may perhaps be connected with the known tendency for individuals susceptible to diphtheria eventually to become immune, doubtless because of occasional exposure to larger or smaller doses of the diphtheria bacillus. Spontaneous development of natural immunity to pertussis or pneumonia does not seem to be nearly so regular; just why is not clear.

Artificial immunity to smallpox can probably be relied on for ten years or so; we have already discussed possible reasons for the greater duration of immunity to many virus diseases. Even in individuals who lose their artificially induced immunity, however, and eventually contract the disease, it is likely that some residual immunity, perhaps only an ability to "tool up" more rapidly for a new job of antibody production, remains, and in many cases results in the attack's being a relatively mild one. Our tissues, like the elephant, remember.

8. Passive Immunization and Serum Treatment

It may be supposed that treatment with antisera is likely to be most successful in diseases where the antibodies normally play a particularly important role. The type of infection, the ease with which the organisms can be reached by antibodies introduced into the blood stream, and the production or nonproduction of a toxin which can be neutralized by antibody are also deciding factors. Even if all of these factors are favorable, serum therapy will not ordinarily be practicable unless we are able to produce a potent antibody to the infectious agent (or its toxic products) in some convenient animal. Recent work with sulfanilamide and related compounds suggests also that as progress is made in chemotherapy, serum treatment may assume a more minor role, at least in certain diseases. However, in pneumonia, even with the use of chemotherapy, recovery of the patient appears to depend in part on whether or not type-specific antibodies are produced (12, 87). The observation that certain strains of streptococci are, or have become, sulfa resistant or penicillin resistant also suggests that antibodies, with their almost infinite adaptability, will continue to be important (82, 95).

Serum therapy has been applied with considerable success in pneumonia, to take a specific example. Antipneumococcus sera have been found to contain agglutinins, precipitins, opsonins, and bactericidins, as well as substances having protective, curative, neutralizing, and immobilizing power for the pneumococcus. Probably all or nearly all of these are simply different manifestations of the activity of one and the same antibody (page 31). After such serum is injected, the extent of *in vivo* phagocytosis, and the clearing mechanism in general, are markedly enhanced, and the blood stream is rapidly sterilized (39).

Treatment of diphtheria with antitoxin was one of the first great triumphs of immunology. It is customary to speak of this as a form of serum therapy, but in a sense the action is prophylactic. A patient's life is saved by antitoxin only if enough is introduced to neutralize the toxin not already fixed by the tissues and that which will continue to be formed in the lesion. Diphtheria toxin is a potent tissue poison, and it appears to produce irreversible injuries, so that after it has united with the tissues no amount of antitoxin will cure these effects.

Passive immunization by the injection of antitoxin is transitory, and to be recommended only when contacts cannot be kept under observation. In such cases the administration of 500–1000 units of antitoxin to each susceptible (Schick-positive) child has been found effective in stopping the spread of the disease.

Another example of the successful use of antitoxin is found in the prophylaxis of tetanus by the administration of tetanus antitoxin. Since passive differs from active immunization in that protection begins at once (and also because it is not lasting), the serum is not given until after an injury which might result in tetanus. It is of course not always easy to decide what injuries are thus to be classified, as tetanus may follow very minor wounds. Zinsser, Enders, and Fothergill (96) state certain general rules: antitoxin should be given following wounds such as compound fractures, gunshot, penetrating, and puncture wounds, machinery wounds, deep cuts; especially if dirt, clothing, splinters, etc., have been carried into them; or after less serious wounds if they may have been contaminated by dirt containing horse manure.

The introduction of active antitetanus immunization with toxoid (see page 384) will doubtless make the use of antitetanus serum, which resulted in a considerable proportion of cases of serum sickness, much less common in the future.

Antianthrax serum has been found useful in treating the human disease, especially in the treatment of cutaneous anthrax (malignant pustule). An initial dose of 100–150 cc. is given intravenously, and 10–15 cc. injected around the area of infection every four hours (3, 8, 60). According to Topley and Wilson (90), antianthrax serum contains neither bactericidins nor antitoxins, and the agglutinin titer is said to be no higher than that of normal serum; it has been claimed that it contains opsonizing and complement-fixing antibodies. The reason for its curative power is not definitely known.

In whooping cough we have a disease in which it has not proved possible to demonstrate that circulating antibodies have anything to do with immunity (29), nor has absolutely convincing evidence been offered that convalescent serum is or value in the treatment of the disease. Zinsser, Enders, and Fothergill (96) point out that, since pertussis is in a sense largely a "surface infection," it is not to be expected that circulating antibodies, even if introduced, would be able to reach the locus of infection in concentration high enough to be effective.

Prophylaxis of certain virus diseases by administration of antibody is successful (see page 386). Serum treatment of virus diseases has been mainly restricted to the use of convalescent serum to prevent or lessen the severity of an attack. Because of the difficulties of obtaining and working with pure virus, it has been difficult to prepare suitable sera in animals, and in addition it is doubtful if antibodies artificially introduced into the circulation would have much therapeutic effect, in view of the fact that the virus is located inside the affected cells (see Chapter IX).

As an example of the use of convalescent serum, we may mention measles. It is desirable to prevent an attack of measles in children under three years of age, or in children in poor health. In other children it is better to aim rather at an attenuation of the severity of the attack. It has been found possible to accomplish these aims by the use of serum from children recently (seven to ten days) recovered from measles, or by use of normal adult serum, placental extract, or concentrated human γ globulin. These preparations all contain antibodies to the measles virus, the convalescent serum the most and the adult serum perhaps the least. Permanent immunity seems to follow an attack thus attenuated, but if the disease is entirely prevented the patient becomes susceptible again after the passive immunity disappears (three to four weeks).

As by-products of the fractionation of human plasma, which was carried out on a large scale during the war to produce human albumin for use as a blood substitute, globulin concentrates rich in various antibodies usually present (presumably as the result of previous attack or exposure) in human serum have become available (18). A γ globulin concentrate has proved highly successful in the prophylaxis and treatment of measles (46, 59) and in the prophylaxis of infectious hepatitis (86). Preliminary studies indicate some value in the prevention and therapy of scarlet fever (46).

Without attempting to summarize fully our knowledge of the application of immunological methods to human disease, we may give a rough idea of the situation in Table XLIII, which shows certain important diseases, some of which can be successfully prevented or cured by immunological methods, and some of which can not. The blank spaces in the table serve to emphasize our ignorance and suggest possible lines of future development. Chemotherapeutic measures, which are successful in a number of these diseases, are not shown.

9. Serum Treatment of Snake Bite

The therapeutic use of antisera is not restricted to bacterial and other diseases, but such therapy has also been applied in cases where the toxic agent comes from reptiles or venomous insects. Injection of antivenin (serum of horses injected with snake venom) has been found effective in the treatment of bites of poisonous snakes. According to Do Amaral (2) this is particularly true in this country, since the North American snake venoms act slowly. Do Amaral thinks that if antivenin is used within 12 to 24 hours the chances of its being effective are good.

Snake venoms exhibit serologically a good deal of species specificity, so that an antivenin for one species may not be effective against another. However, an antivenin against a related species is usually

TABLE XLIII[a]

IMMUNOLOGICAL METHODS IN PREVENTION AND TREATMENT OF CERTAIN DISEASES

BACILLARY DISEASES

Disease		Prevention and treatment by					
		Vaccine	Toxin or toxoid	Anti-bacterial serum	Anti-toxic serum	Human immune serum	Concentrated human globulin
Tuberculosis	P	+					
	T						
Diphtheria	P		+				
	T				+		
Tetanus	P		+				
	T				+		
Botulism	P						
	T				+		
Gas gangrene	P	+?					
	T				+		
Dysentery	P	+?					
	T			+			
Anthrax	P						
	T			+			
Typhoid and paratyphoid	P	+					
	T						
Cholera	P	+					
	T						
Plague	P	+?					
	T						
Whooping cough	P	+				+	
	T					+	
Tularemia	P						
	T						
Leprosy	P						
	T						
Brucellosis	P						
	T						

+ = successful application. − = lack of success. P = prevention. T = treatment.

[a] Based on a chart published in *Life*, November 5, 1945.

TABLE XLIII (cont.)

Disease		Prevention and treatment by					
		Vaccine	Toxin or toxoid	Anti-bacterial serum	Anti-toxic serum	Human immune serum	Concen-trated human globulin
COCCUS DISEASES							
Streptococcus infections	P	+					
	T			+?			
Staphylococcus infections	P	?					
	T	?					
Pneumonia	P	+[b]					
	T			+			
Meningitis	P						
	T			+			
Gonorrhea	P						
	T						
RICKETTSIAL DISEASES							
Rocky Mt. spotted fever	P	+					
	T			+			
Epidemic typhus	P	+					
	T			+			
Scrub typhus	P						
	T						
Murine typhus	P	+?					
	T						
PROTOZOAN DISEASES							
Malaria	P	−					
	T						
Amoebic dysentery	P						
	T						

+ = successful application. − = lack of success. P = prevention. T = treatment.

[b] Group specific polysaccharides.

Table XLIII (cont.)

Disease		Prevention and treatment by					
		Vaccine	Toxin or toxoid	Antispirochete serum	Antitoxic serum	Human immune serum	Concentrated human globulin
SPIROCHETE DISEASES							
Syphilis	P						
	T						
Yaws	P						
	T						
Infectious jaundice	P						+
	T			+			
VIRUS DISEASES							
Influenza	P	+					
	T						
Smallpox	P	+					
	T						
Rabies	P	+					
	T						
Encephalitis	P	+[c]					
	T						
Mumps	P	+?					+
	T						+
Measles	P					+	+
	T						+
Yellow fever	P	+					
	T						
Lymphogranuloma venereum	P						
	T						
Ornithosis	P						
	T						

+ = successful application. – = lack of success. P = prevention. T = treatment.

[c] Effective in only one of the six known types.

of some help. The venoms of the various North American rattlesnakes, for example, are almost identical serologically. Antivenins which are at least somewhat effective are now available for most of the commoner species (96) of poisonous snakes.

TABLE XLIII (concluded)

VIRUS DISEASES (cont.)

Disease		Prevention and treatment by					
		Vaccine	Toxin or toxoid	Anti-virus serum	Anti-toxic serum	Human immune serum	Concentrated human globulin
Chicken pox	P						
	T						
Common cold	P						
	T						
Poliomyelitis	P					+?	+?
	T						
Dengue fever	P						
	T						
Atypical ("virus") pneumonia	P						
	T						
Infectious hepatitis	P						+
	T						+?

+ = successful application. − = lack of success. P = prevention. T = treatment.

10. DIAGNOSIS

Since in the present book we are concerned with *immunity* and *immune mechanisms* solely, we shall say nothing about the applications of serological methods to the *diagnosis* of diseases, but leave that topic to those more interested in clinical medicine. We may merely remark that of course immunological methods have considerable value in diagnosis, whether in determining serologically the type of infectious agent present by tests on the isolated organism, by test of the patient's serum for antibodies against a suspected micro-organism or virus, by tests for antibodies not specifically directed towards the invader, but produced as a sort of by-product of the infection, or by skin tests for susceptibility or allergy. Examples of some of these technics will be found in the next chapter (see *Pneumococcus* typing, Kahn and Wassermann tests, Widal test, Schick test, tuberculin test, etc.).

11. THE CONTROL OF COMMUNICABLE DISEASES

A short but useful summary of this subject, covering many diseases not mentioned here, will be found in a book issued by the American Public Health Association (4).

Appendix: Skin Tests for Susceptibility and Immunity

The reader may have noted, possibly with some slight confusion, that skin tests are frequently employed in experimental and clinical immunology, but that the results may vary considerably in characteristics and significance. Until we had finished the discussion of allergy of infection given in the preceding chapter, it was impossible to give an intelligible account of skin tests in general without anticipating much later material. We may now pause to consider the types of skin tests and their significance.

The skin, once thought of as a mere covering, is now known to be an important organ, having a mass twice that of the liver, and many indispensable functions. It is an immunological organ too, and, whether or not it can form antibodies, takes part in many important immune reactions. Some of these seem to be peculiar to the skin, as in eczema and contact dermatitis, others merely reflect the state of the body tissues in general; and the reaction takes place in the skin, which forms the first line of defense against the entry of many foreign substances, because, for convenience, we introduce the material into the skin artificially, or it is naturally deposited there.

Essentially, skin tests are fitted to reveal the presence or absence of two different conditions, immunity and hypersensitiveness. However, it will now be appreciated that hypersensitiveness is itself fundamentally immunological in nature, so the two phenomena are not unrelated. Also, we have seen above that there is good reason to suppose that the hypersensitiveness of infection, at least, is to some extent an indication of immunity. The choice, in any given case, of one or the other of the above terms depends largely on the kind of reagent used for the test.

In the test for immunity, we make use of a toxic product from the disease-producing agent. In normal, non-immune individuals this will cause damage to the tissues where it is injected, and a reaction, accompanied by redness, itching, and perhaps necrosis, will follow its introduction. This reaction will take time to develop, as the deleterious effects of toxins and similar substances are not immediately apparent. The immune individual, probably because of the presence of free antibody in his skin or circulation, does not react in this way; the toxin, in the concentration used, is neutralized by the antibodies, and either no lesion, or only a mild one, is produced. Tests of this sort are the Schick test for diphtheria and the Dick test for scarlet fever, using diphtherial and scarlatinal toxins, respectively.

In tests for hypersensitiveness, we employ materials which are harmless to the *normal* person, so that a positive reaction is obtained in the susceptible individual. We must remember that there are two types of hypersensitive reactions, in so far as the skin is concerned. In the first, exemplified by atopic hypersensitiveness, the reaction following the introduction of the allergen is almost immediate, developing within a few minutes. This is in line with the known speed of serological reactions, and the hypothesis that the mechanism of the reaction is the union of antigen and antibody, liberating histamine or a histamine-like substance. Reactions of this kind are characteristic of many allergic conditions where no infection with a living agent is involved, such as hay fever, but they are also observed in a number of diseases, as with the specific polysaccharide in pneumonia, and in a number of parasitic and fungus infestations. Reactions of the immediate type to diphtheria toxin and to tuberculin have also occasionally been observed.

In a number of bacterial infections, such as tuberculosis and brucellosis, the reaction to the introduced material is of the delayed type, developing in about 24–48 hours. The reason for this delay is not understood, but the reaction nonetheless denotes the presence of a hypersensitiveness. Enders (24) has found that a skin sensitivity to virus develops after recovery from mumps, and on the whole correlates well with a state of resistance.

Since an animal will never be naturally hypersensitive to constituents of an infectious agent without having actually been infected, the presence of such a hypersensitiveness implies a history of infection. Thus the chief value of this test is in diagnosis. In a number of cases it has been found that the more acute the hypersensitiveness, the higher the resistance of the host. Therefore, in such cases the degree of reaction obtained in the skin test is some indication of the degree of immunity. Either the allergic mechanism is essentially part of the immune process, as suggested above, or development of an allergy to a microorganism or its products tends to parallel the development of immunity.

We may sum up the situation in Table XLIV.

It has been found that the type of skin hypersensitiveness giving the immediate type of reaction can be transferred passively (see page 329). The procedure is called the Prausnitz-Küstner (66) technic,

TABLE XLIV

SIGNIFICANCE OF SKIN REACTIONS IN DIFFERENT CONDITIONS

Type	Reagent	Type of reaction	Reason for reaction	Character of reaction	Meaning of a positive reaction (erythema, wheal, itching, etc.)
Schick, Dick.	Toxin.	Delayed.	Damage to tissues by toxin.	Inflammatory.	Absence of immunity (= absence of antibody).
Atopic (urticarial).	Antigenic extract.	Immediate.	Antibody–antigen reaction.	Wheal, erythema.	Antibody (reagin) due to hypersensitiveness, in some cases to infection.
Tuberculin.	Antigen from microorganism.	Delayed.	?	Inflammatory.	Hypersensitiveness due to infection. Some immunity?
Contact.	Various nonantigenic excitants of contact dermatitis.	Delayed.	?	Eczematous.	Hypersensitiveness.

after the men who first reported it; details will be found in Chapter XI. The delayed type of skin reactivity apparently can not be transferred.

The significance and mechanism of these various skin tests has also been discussed by Janeway (45).

SUMMARY

(*1*) Immunological methods have not been developed to the point at which all human diseases can be prevented or cured. (*2*) It is not always clear why immunological methods are more successful in some cases than in others: (*a*) some diseases seem intrinsically to produce less immunity than do others, (*b*) different strains of a pathogen may exist to cause the same disease (or one clinically indistinguishable from it), (*c*) people vary individually in their ability to build up immune resistance, (*d*) in some diseases immunological methods are not as helpful and important as other measures, (*e*) immunization is probably most successful in diseases in which antibodies play an important role, (*f*) cultivated strains of pathogens may sometimes lack an important antigen, (*g*) some pathogens contain better antigens than do others, (*h*) sensitization produced by attempts to immunize may be a disadvantage (page 371), (*i*) immunization will not usually be applied routinely in the case of rare or mild diseases. (*3*) Antibacterial immunity produced by an attack of the disease results from intimate contact with large numbers of microorganisms containing their full complement of antigens. In artificial immunization we have to give doses of limited size, at intervals, and sometimes the organism utilized lacks an important antigen. Nevertheless, in a number of cases the results are well worth while (e.g., typhoid, pertussis, and pneumonia). (*4*) Antitoxic immunity is important when the pathogen produces a potent exotoxin. Detoxified toxins (toxoids) produce good immunity in several cases (e.g., diphtheria, tetanus, and gas gangrene). Injection of scarlatinal toxin protects against scarlatinal rash, and, according to many authorities, against scarlatinal streptococcus infection. (*5*) Immunity to virus diseases following an attack is often nearly lifelong; this may be because (*a*) such viruses may be very potent antigens, possibly because they are brought into more intimate contact with the tissues of the host (being intracellular), (*b*) the immunity resulting from infection may be reinforced by later contacts, and (*c*) living virus may in some cases persist in the apparently fully recovered host (as in herpes simplex). (*6*) We may obtain active antivirus immunity by provoking an attack of the disease, but methods are available which often enable us to avoid this risk. One of the oldest is the introduction of the (fully active) virus through an unnatural portal of entry (e.g., variolation). A later, but still old, procedure employs an "attenuated" virus (e.g., vaccination with the virus of cowpox). Injection of killed viruses is usually successful if sufficient material is introduced (e.g., influenza). (*7*) Artificial immunization probably does not as a rule give immunity as solid and lasting as does an attack of the disease, because smaller amounts of antigenic material are involved, and we may employ material not containing all of the antigens produced by the complete organism. However, immunity can usually rather easily be renewed. (*8*) Passive treatment with antisera seems to be most successful in diseases in which antibodies normally play a particularly important role, and in which the invading microorganisms are readily reached by the circulation. Treatment with antiserum has been successful in diphtheria and pneumonia, for example. Passive immunization with serum confers only a transitory immunity, but has proven worth while in tetanus (although increasingly displaced nowadays by active immunization). Concentrates of "normal" human antibodies from pooled plasma have been shown to have potency in preventing and treating measles, in preventing infectious hepatitis, and possibly in prevention and treatment of scarlet fever. A summary of applications of immunological methods to important diseases is given in Table XLIII. (*9*) Antisera to the venoms of certain venomous animals (such as the rattlesnake) have been found effective. (*10*) Immunological methods have important applications in diagnosis (*Pneumococcus* typing, Kahn and Wassermann tests, Widal test, Schick and Dick tests, tuberculin test, etc.). (*11*) The control of communicable diseases is summarized in a booklet issued by the American Public Health Association (4). *Appendix:* The mechanism and meaning of various skin tests for susceptibility and for immunity are discussed, and are summarized in Table XLIV.

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CHAPTER XI

LABORATORY AND CLINICAL TECHNIC

A. Apparatus

A good electric centrifuge and an electric refrigerator or cold room are perhaps the most important and essential features of a serological laboratory; a water bath is also practically indispensable. The most useful type of centrifuge will probably be found to be the Size 2 centrifuge manufactured by the International Equipment Co., which can take care of very small as well as fairly large quantities. Cups plus tubes should be carefully balanced, and balanced pairs should be placed opposite each other in the machine. Centrifuge tubes holding about 100 cc., made of Pyrex glass, should be available, with a head and cups of suitable size. For smaller quantities test tubes of the "Wassermann" type, or smaller, may be used, in smaller cups interchangeable with the larger ones.

Test tubes of the "Wassermann" type, roughly 100 × 13 mm., holding 8–9 cc., are suitable for a large number of tests. Occasionally larger tubes may be needed. For blood grouping tests, tubes measuring about 75 × 9 mm., holding about 4 cc., also without lip, are desirable. For the performance of "ring" precipitin tests, "micro" tubes specially made from semicapillary tubing are desirable to avoid waste of material (see page 421). On occasion, graduated centrifuge tubes, holding about 15 cc., will be found useful. It is desirable if possible to have all test tubes made of Pyrex glass. For standardizing bacterial suspensions, and occasionally for measuring volumes of precipitates, the "Hopkins tube," which consists of a small, graduated tube forming the lower portion of a centrifuge tube of about 10-cc. capacity, will be found useful. For optimal proportions determinations the present author prefers small tubes without lip, holding as nearly as possible 1 cc., and all of the same internal diameter.

Test tube racks, adapted to holding Wassermann tubes, will be desirable. The size may depend upon the individual preference; a type holding two rows of six or eight tubes each will be found useful. For other purposes, such as blood grouping, galvanized racks similar to the "Kolmer" type, holding four rows of 12 each, are better. For some of the smaller types of tubes it may be necessary to make suitable racks or supports. For larger tubes, such as the large centrifuge tubes, galvanized wire baskets, preferably of the square or rectangular type, are useful.

Several types of pipets will be needed. Some of the ordinary chemical volumetric pipets, in sizes of 5, 10, and 20 cc. will be useful, but, in addition pipets of the serological type (graduated to the tip), in sizes of 1, 2, 5, 10, and (sometimes) 15 cc. will be wanted. For certain kinds of work other types, for instance a 0.2-cc. pipet graduated in hundredths, may be desired. So-called capillary pipets, made by drawing out to a fine tip (about 1 mm.) capillary pieces of ordinary (7–8 mm.) laboratory glass tubing, used with rubber bulbs, will be almost indispensable. These are harder to make if Pyrex tubing is used, but the product is more durable.

For many kinds of work sterile pipets are needed. Before sterilization, cotton is stuffed in the upper end of the pipet, not too tightly, and the excess is burned off by quick passage through a flame. They are sterilized in the hot air oven in copper cans, preferably of the rectangular type, each size in a separate can.

For sterilization of serum, etc., where heating would be deleterious, and where it is not desired to add a preservative, filters of the Berkefeld or Mandler type will be useful. The Berkefeld "N" or Mandler "ordinary" are usually suitable. These are used in connection with a sterile side arm filter flask (of Pyrex) holding about 250 cc. A water (or mechanical) pump to provide suction is almost essential (see, however, 18). Seitz filters are often more convenient, since they can be prepared rapidly, and the filter pad is discarded after each use. Sizes adapted to very small as well as quite large amounts are obtainable. When possible the kind employing positive pressure instead of suction should be used.

As a preservative of biological materials, "Merthiolate" (Lilly), or phenyl mercuric nitrate in a concentration of about 1:5000 to 1:15,000, are widely used. For preserving as well as marking blood grouping sera, certain dyes (acriflavine, brilliant green) have been found useful (13, 106). For the preservation of erythrocytes for agglutination tests, the mixture of Rous and Turner (107) is suitable, but the newer ACD (acid citrate dextrose) mixtures are apparently even better (78, 79). For material which is to be injected, phenol to 0.5% has been found satisfactory.

It is important that all glassware be scrupulously clean. Glassware is much more easily cleaned if it is immersed in water immediately after it has been used. When infectious material has been used, 1% formalin should be used. When pipets become greasy inside, ordinary washing is not sufficient, and they should be soaked 24–48 hours in a solution of sulfuric acid and dichromate (for instance, 100 g. potassium dichromate, 1 l. sulfuric acid, 200 cc. water). Cloudy glassware should never be used, unless it is ascertained (by treatment with this cleaning solution) that the cloudiness is due merely to etching of the glass.

TABLE XLV

TYPES OF HYPODERMIC NEEDLES NEEDED FOR DIFFERENT PURPOSES[a]

Purpose	Length (inches)	Gage
Subcutaneous or intra-abdominal injection of viscous material, and bleeding rabbits from the heart	1.25	18
Other subcutaneous or intra-abdominal injections in animals	1	20
Single intravenous injection into rabbits	0.75	23
Intracutaneous injection into human beings, or repeated intravenous injection into rabbits	0.50	25, 26, 27
Intracutaneous injection into guinea pigs	0.50	27, 28
Bleeding sheep from the jugular or large dogs from the heart	3.50	14
Bleeding human beings from the arm veins	1	18, 20
Subcutaneous injection of large volumes into guinea pigs	1	23
Intramuscular injection of human beings	1.50	21

[a] Modified from Coca (23).

Two types of syringe will be needed, the Luer all-glass (Pyrex) in sizes of 2, 5, 10, 20, 30, and 50 cc., and the "tuberculin" 1-cc. syringe. Schick-test syringes (1.0 cc. graduated in tenths with graduations encircling the syringe) are better than the tuberculin type for all except the most minute measured injections. Needles of various sizes will be needed, as shown in Table XLV. Stainless steel needles are the best on the whole; they may be sterilized by boiling or autoclaving. Platinum needles, although harder to keep sharp, are preferred by some workers who make many injections in the course of a single day. The ends of platinum needles can be sterilized by heating to redness in a flame, a procedure which is rapid and sometimes convenient; it would of course destroy the temper of steel needles. Thus the same needle, if sterilized by boiling in the morning, may be used for skin tests throughout the day.

For injecting or bleeding guinea pigs, a holder consisting of a board with numerous holes in it, plus a carrier holding a cross bar for the head, is useful. The animal's legs are tied down with loops of rawhide. This holder is described and illustrated by Coca (23).

For rabbits various holders have been recommended, from a plain towel to a box with a sliding cover which has a notch for the neck, in which the animal is placed with the head outside (23). The present writer has found it perfectly feasible, and on the whole simpler, to hold the rabbit on the lap (which is protected by a towel or thick laboratory coat) while drawing blood from the ear or injecting into the ear veins. The animals soon become accustomed to sitting quietly during these procedures, which cannot be very painful. In this way a single person can carry out the operation. For drawing blood from the heart an assistant is necessary, and is helpful when injecting intra-abdominally, although this too can be accomplished by one person who knows how to hold the animal between the knees. The assistant holds the animal firmly by the hind feet in the left hand, and holds front feet and neck in the right hand, using his knees to prevent motions by the animal.

B. Technic of Injection and Bleeding of Animals

1. Injection

Rabbits are the animals mainly used for the production of experimental antisera, and serum containing antibodies (antiserum) from rabbits possesses advantages (lower molecular weight, lower solubility = greater precipitating power) over that from horses, and possibly over that from chickens, which are sometimes used. Other animals, such as guinea pigs, are too small, and furnish too little blood, for common use as precipitin producers, and may not produce so well anyway.

A number of rabbits (three to five) should be given each type of antigen used, as one or more may die during the injections, and not all rabbits produce good antisera to any given antigen. It is probably best to inject the antigen intravenously (except in antitoxin production in the horse), using other methods only as supplementary to this.

Before starting the injection a syringe (a 2-cc. or 5-cc. type is suitable) and needles (probably 25-gage is best although there are a few who prefer larger sizes) are boiled 20 minutes to sterilize them. Modern syringes may be boiled assembled.

The injection is made into the external marginal vein of the rabbit's ear. During this procedure many rabbits will sit quietly in the operator's lap, others may require a "rabbit box" or rolling in a towel (see page 400). If the vein is hard to see, or if difficulty is experienced by the operator in inserting the needle into the vein, a *little* xylene may be rubbed on the ear with cotton, then washed off with alcohol. (In all cases a little alcohol, by laying the fur, makes the vein more visible, and by its use the necessity of shaving the ear is avoided.) If the vein does not come up, a little rubbing with dry cotton will now bring it up. The xylene and rubbing, essential for bleeding (see page 402) are not usually necessary for injecting. The needle is inserted into the vein, in the same direction as the circulation (towards the heart), the ear and needle held with the left hand so that the thumb is over the point of entry, and the antigen injected, slowly at first. Resistance or visible distention of the tissue around the vein indicates that the needle is not in the vein. Any injection into the perivascular tissue is likely to destroy the future usefulness of the vein for injection.

One ear (right or left) is always used for the injection, the other being reserved for bleeding. Injections at the beginning of a course are made near the tip of the ear; bleedings begin near the root.

In some cases intra-abdominal injections are desirable. The rabbit is held, belly outwards, between the operator's knees, and the head and forefeet are held with the left hand. Enough alcohol to wet a small area of fur is dropped on the belly, the (20-gage) needle of the already charged syringe is inserted, first through the skin, then through the abdominal wall, and the injection is made with the right hand. When dealing with infectious material, scrupulous asepsis should be observed.

2. BLEEDING

Blood may be taken from the rabbit's heart, with a 50-cc. syringe and large (18-gage) needle, but occasionally an animal will die. Almost as much blood may be taken, not so quickly, but with less risk, from the marginal vein of the ear, if the technic described under *Injection* (see above) is used to engorge the vein. A longitudinal (about 2 to 3 mm.) slit is made in the vein with a sharp scalpel; the blade of a Bard-Parker knife is convenient. If a clot forms in the incision it should be scraped off. If the flow stops, another treatment with a little xylene, and friction, may revive it. In this way 50 to 80 cc. of blood may be taken. Similar bleedings may be made on the day following, and the day after that. Further bleeding without a rest period of several days (and preferably preceded by another injection), may result in the death of the animal.

If it is desired to bleed the animal to death, most blood is obtained by inserting a canula in the carotid or femoral artery.

3. Dosage

There is no apparent advantage in giving more than 1 or 2 cc. of a 1% (protein) antigen at each intravenous injection. Intra-abdominal injections should be about three times as large. It is the practice of many to increase the size of the dose, particularly of non-toxic antigens, as immunization proceeds, but it is not certain that any better antisera are thus obtained.

4. Injection Schedule

There is great individual variation in the procedure followed by different workers, and no experiments sufficiently elaborate to determine what is best have ever been done. The animal may be injected intravenously once, then after six to eight days intravenously again, then after six to eight days intra-abdominally, followed by a trial bleeding in 10–14 days (Uhlenhuth). The technic of the present writer is as follows: intravenous injections the first three days of one week; one intra-abdominal and two intravenous injections the first three days of each of the following three weeks, followed by a trial bleeding one week after the last injection. Good antisera have been obtained by both of these, and by a variety of other methods (see 13). Exclusively intravenous injections of small doses of (expensive) antigens have furnished good antisera. Injection of alum-precipitated antigens is recommended by some workers.

If an animal is refractory and requires an unusually long course of injections, the antiserum obtained may be less specific than that from other animals. It is better to discard such animals.

No matter what the exact schedule followed, after a few injections have been given, later intravenous injections, especially if the antigen consists of cells, coming after the lapse of more than one day, should be prefaced by an intra-abdominal injection on the preceding day, to avoid possible anaphylactic shock.

5. PRESERVATION OF ANTISERUM

If blood is taken from the heart with a *sterile* needle and syringe, it will be sterile, otherwise it may not. If it is not, the serum should be treated for preservation, preferably by passage through a Berkefeld or Seitz filter, followed by storage in a sterile container in the icebox. Addition of "Merthiolate" (1:5000) may be satisfactory. Phenol is likely to cause clouding and make sera anticomplementary in complement fixation tests.

6. INJECTION OF HORSES

This is not a procedure likely to be undertaken often in any laboratories except those routinely producing commercial therapeutic horse antisera. A description of the methods used at the Massachusetts State Antitoxin Laboratory will be found in (134). See also Wadsworth (122).

The technic recommended in (134) for producing diphtheria antitoxin involves the subcutaneous injection of toxoid, on opposite sides of the horse, at intervals of two to three days, starting with 5 cc., and working up to 200 cc. Then subsequent injections are given of toxoid plus calcium chloride, starting with 50 cc., and up to 200 cc., if temperature reactions permit. The first bleeding of 7–8 l. is usually taken about the twenty-fifth day of immunization, directly into citrate.

C. Preparation of Materials for Clinical Use

1. CONVALESCENT SERUM

Such serum has been found useful in treating scarlet fever or in protecting for a short time against diseases such as measles, pertussis, and possibly poliomyelitis.

In case only a small amount (5–10 cc.) of serum is desired, the blood may be taken in the usual way with a 50-cc. syringe. It is assumed that anyone attempting this has had previous experience in taking blood. We shall not attempt to describe the process in words, for the only practical way to learn the technic consists in seeing it done. Briefly, the procedure consists in slight traction of the plunger while the needle remains in the vein, the tourniquet being left on until just before the needle is removed.

The skin over the vein should be treated with tincture of iodine followed by alcohol, and all steps performed with strict aseptic precautions. The blood is transferred to a sterile, stoppered test tube (such as a 100-cc. centrifuge tube) and the serum allowed to separate over night. As soon as the blood has clotted it is advisable to separate ("rim") the top of the clot from the side of the tube by use of some sterile instrument such as a glass rod. The serum is poured off next morning into a sterile tube, centrifuged to remove erythrocytes, and placed in a suitable container.

For larger amounts of serum, blood may be collected, either into sterile 100-cc. centrifuge tubes, or (if a suitable head is available for the centrifuge) into larger tubes, by inserting into the vein an 18-gage needle connected to a three-inch piece of soft rubber tubing, the whole having been sterilized by autoclaving. This procedure is described with illustrations by Kolmer (69). Use may be made of a prepared sterile assembly consisting of a 500-cc. Erlenmeyer flask, a two-hole rubber stopper fitted with two pieces of bent glass tubing, rubber tubing attached to these, and an 18-gage needle attached to one of the pieces of rubber tubing. The needle and piece of tubing without needle for suction are protected during sterilization and until ready for use by test tubes plugged with cotton. The bleeding technic used by the American Red Cross during the war has been illustrated by numerous advertisements and folders. Serum is removed in such procedures by use of sterile pipets. Before any of it is injected it should be cultured to test for sterility and tested for syphilis.

Preparation of convalescent serum requires experience, and fatalities have resulted from use of material prepared by inexperienced personnel. A colleague informs me that it is now illegal in at least one state of the union unless done in a laboratory licensed for that purpose by the state.

2. Preparation of Vaccines (Bacterial)

The preparation of all bacterial vaccines is essentially similar. When they are to be used for prophylactic immunization, the organisms are selected from a stock strain, or perhaps, better, a recently isolated strain known to have high virulence. They should be grown on suitable media. Certain strains have become classical, although modern work has in some cases indicated that during the years they have grown on laboratory media they have lost important antigenic constituents (see page 371). When vaccines are to be used for treatment of an existing infection they are often made by growing and killing organisms actually isolated from the patient (autogenous vaccines).

When procuring the infected material, precautions should be observed against contamination by other organisms not responsible for the infection. An appropriately stained smear should be examined. Of course disinfectants can not be added to the material, or brought into too close contact with the tissues, without danger of killing the responsible organism. In general the infected area should be cleansed as well as possible, and sterile apparatus should be used in collecting the material.

The next step is the preparation of a pure culture. If this is not obtained when the infected material is cultured (usually on a blood plate), it may be necessary to employ the usual bacteriological technics for separating the organisms, and to have a knowledge of the patient's condition when selecting the organism or organisms for the vaccine.

It is usual to grow organisms which are to be made into a vaccine on solid media, then to wash them off in saline and kill them, first shaking thoroughly to break up clumps. If the emulsion obtained is not perfectly homogeneous, the larger particles should be removed by brief centrifugation or filtration through a sterile filter (glass wool may be used). The vaccine is usually killed by heat, using the minimum temperature for the minimum time (e.g., 56–60°C. for one hour). In some cases this is too drastic a procedure for some of the important antigens present (see page 155). Then the vaccine is cultured to test for sterility. At least a dozen loopfuls are transferred, with strict aseptic precautions, to a slant of a suitable medium, which is then incubated for 24 hours, or longer if the organism is one that grows slowly.

It is desirable to make at least two tests for sterility, one suitable for aerobic growth, one for anaerobic growth. The dead organisms should be washed before the tests are made, and the tests made before the addition of any preservative, to avoid possible bacteriostatic effects due to preservatives.

If the vaccine is sterile, it may next be standardized. The concentration of microorganisms is determined and the vaccine is diluted with sterile saline to proper strength. The concentration may be determined by actual counting in a hemocytometer or special counting chamber, by centrifuging a measured volume in a Hopkins tube, or by turbidimetry. From percentage concentrations obtained in the latter way, it is possible by tables to convert with sufficient accuracy, to approximate numbers of organisms per cubic centimeter (see 69). The average vaccine is likely to be made to contain from one-half to one billion organisms per cubic centimeter (roughly a 0.1% suspension). Sometimes two strengths, a weaker for initial, and a stronger for later, injections are prepared.

After the vaccine has been diluted, phenol to give a final concentration of 0.5% is added as a preservative and to guard against accidental contamination. This is very important, especially if the vaccine is being kept in bulk. The vaccine is put up in sterile bottles of suitable size, fitted with sterile rubber stoppers having a thin place to allow insertion of a hypodermic needle.

Descriptions of the technic of preparing vaccines, with details of the methods appropriate in particular cases, will be found in (69, 122).

3. Preparation of Smallpox Vaccine Virus

This will not be described in any detail here, as it is not a procedure likely to be attempted in the laboratory, and the vaccine is readily available commercially. Methods of preparing it are described by numerous authors (see 69, 134). In the calf method, still commonly used, the abdominal skin of a healthy young heifer is shaved and thoroughly cleansed. Parallel scratches 1–2 cm. apart are made over the clean area, and the virus (which is occasionally passed through rabbits to retain virulence) is rubbed into these. Rigid cleanliness is observed during and after the inoculation. The virus is harvested on the sixth or seventh day, after thorough cleansing of the abdomen. The pulp obtained is ground with glycerine, and allowed to age so that bacteria, which are usually present, may be killed by the glycerine.

The finished product is tested for purity (*a*) by plating and counting colonies grown at 37°C. and 20° (*b*) by observing growth and gas in 2% glucose broth (*c*) by incubating anaerobically and testing any growth for tetanus by injecting mice or guinea pigs and (*d*) by injecting guinea pigs and observing ten days for evidences of streptococcal infection. Before the product is released it must be free of contamination with *Clostridium perfringens*, *Cl. tetani*, and streptococci.

Vaccinia vaccine has been prepared from virus grown on the developing chick embryo. In this particular case the vaccine prepared from the embryos has not proved any better than, if as good as, vaccine prepared in the more usual way. However in a number of cases growing a virus on chick embryos has proved the only practical method. As Goodpasture (39, 128) points out: "The developing egg has proved to be susceptible to a greater number of viruses than any other living host."

Influenza vaccine prepared from growth on chick embryos was developed during World War II, and is now available commercially.

4. Preparation of Diphtheria Toxin

Quite pure toxin in high concentration (of the order of 100 L_f units per cubic centimeter) can be prepared using the synthetic medium of Mueller (90, 92), and, since this avoids the presence of contaminating proteins and peptones, it seems likely that it will displace the other methods of preparation. The medium is particularly adapted to the "Toronto" strain of the Park 8 strain of diphtheria bacillus. The concentration of some of the reagents is very critical, for instance the iron (91), and the formula should be adhered to very closely, using good grades of reagents. The cultures are incubated at 34°C., and the toxin is harvested on the eighth or ninth day, as little increase occurs after this time.

5. Preparation of Toxoid

The National Institute of Health requires that not more than 0.4% formalin be used in the preparation of toxoid; actually 0.3–0.35% is satisfactory (134). Toxin [which should have more than 15 L_f units (see above) per cubic centimeter] which has not been incubated over ten days is mixed with formalin to the desired concentration, and the *p*H, if necessary, adjusted to 8.2. At the end of a 30-day incubation period, and every one or two weeks afterwards, it is tested for toxicity for guinea pigs. When the preparation is finished it must be found that 5 cc. of the toxoid given subcutaneously to a 300-g. guinea pig (at least four animals to complete the test) causes no signs of diphtheria toxin poisoning within a 30-day period. The antigenic power must be at least such that a dose of 0.5 cc. protects, when six weeks have elapsed after injection, at least eight out of ten guinea pigs against five MLD of toxin for ten days or more.

Since diphtheria toxoid is available commercially, probably few laboratories attempt its preparation themselves. Methods are described in (134).

6. Titration of Toxin and Antitoxin

For the principles on which these titrations are based, the reader is referred to Chapters II and VI, pages 69 and 222. The Ramon flocculation titration will be described on page 439, under the optimal proportions titration. The technic here described is from the bulletin written by Rosenau (105). See also (134).

First, preliminary measurements of the MLD and L_+ dose of the toxin are made. (These terms are defined in the text immediately below.) Standard antitoxin, which may be obtained from the National Institute of Health, is needed. The MLD is arbitrarily defined as the amount of toxin which, when injected subcutaneously, causes the death in four days of 75 to 80% of the (250-g.) guinea pigs used. Its determination is illustrated in Table XLVI.

Table XLVI

Determination of the MLD of Toxin (105)

Dose, cc.	Result
0.03	Death in 1.5 days
0.02	Death in 1.5 days
0.01	Death in 2 days
0.008	Death in 3 days
0.006	Death in 3.5 days
0.005	Death in 4 days = MLD
0.004	Death in 6 days
0.003	Death in 8 days
0.002	Late paralysis
0.001	Well in 16 days

This determination is thus essentially an assay with "graded response" (see page 461). The accuracy with which the MLD is determined by such experiments depends of course upon the number of animals used (see page 409). It will be pointed out later that a better end point to have taken would have been the dose which kills 50% of the animals. It will also appear that for accurate determination of the MLD, considerable numbers of animals would be needed, instead of the three or four apparently sometimes used.

Trevan (118) has calculated that, if guinea pigs of the same weight are selected at random, about 50 must be used in each group if the relative toxicities of two samples of diphtheria toxin are to be known with an error of not over ±10%. If a 20% error is allowable, 11 to 12 animals in each group will suffice.

For most accurate work the guinea pigs should be bred and kept under carefully standardized conditions. It has been found that there is some influence of the time of year the tests are done, somewhat less toxin being required to kill a guinea pig in the winter. Corrections to allow for differences in the weight of the animals and differences in time of death have been suggested. For many purposes, however, we do not really need to know the MLD very precisely. A discussion of the errors involved in testing diphtheria toxin for toxicity is given by Glenny (36).

The value of the MLD of toxin is useful in determining the Schick-test dose, etc. It is not needed for titration of antitoxin, but for this determination we do need the value of the L_+ dose of toxin. This is defined as the amount of toxin which, when mixed with one standard unit of antitoxin and injected subcutaneously into a 250-g. guinea pig, causes its death in four days. This determination is illustrated in Table XLVII.

TABLE XLVII

DETERMINATION OF THE L_+ DOSE OF TOXIN (105)
USING "STANDARD" ANTITOXIN

Antitoxin unit	Toxin added (cc.)	Reaction	Antitoxin unit	Toxin added (cc.)	Reaction
1	0.2	No reaction	1	0.26	Fatal in 9 days
1	0.21	No reaction = L_0	1	0.28	Fatal in 6 days
1	0.22	Local infiltration	1	0.29	Fatal in 4 days = L_+
1	0.23	Fatal in 17 days	1	0.3	Fatal in 3 days
1	0.24	Fatal in 14 days	1	0.31	Fatal in 2 days

When a standardized toxin is available, unknown antitoxic sera may be titrated in terms of it, remembering that the toxin is the less stable reagent. An example of such a titration is given in Table XLVIII.

This experiment, according to the original Ehrlich standard, would indicate that the antiserum contains one antitoxin unit in 1/900 of a cubic centimeter, or 900 units per cubic centimeter. Others prefer to base the labelled strength on the amount which just results in survival, so the above serum, on that basis, would be marked as containing 700 units per cubic centimeter.

It is clear that procedures such as this, subject to unavoidable variation in animals, and employing rather coarse steps in the titration, can not be highly accurate, but the method has one important advantage over the Ramon flocculation titration, which otherwise might replace it, in that it gives a direct indication of the actual protective power of the serum, which is of course the all-important factor in therapeutic sera.

The above is but a brief sketch of the actual technic of titration, which can hardly be carried out without reference to more detailed directions (36, 105), and some actual experience.

TABLE XLVIII

STANDARDIZATION OF AN UNKNOWN SERUM

Test dose		Effect on animal
Amount of toxin solution (1 L_+), cc.	Amount of antiserum, cc.	
0.29	1/500	Lives
0.29	1/600	Lives
0.29	1/700	Lives
0.29	1/800	Dies in 8 days
0.29	1/900	Dies in 4 days
0.29	1/1000	Dies in 2 days

D. Laboratory and Diagnostic Procedures

1. DILUTING FLUID

In many serological procedures the directions call at one point or another for dilution of one of the reagents. Unless something else is specially specified, the dilutions are made with a 0.9% (0.15 M) solution of sodium chloride, usually referred to as saline, or physiological salt solution.

2. BLOOD GROUPING

In choosing a donor for blood transfusion, and in testing bloods in cases of disputed parentage, as well as in a number of experimental procedures, it is necessary to determine the blood group of the persons concerned. This is done by allowing the erythrocytes of the individuals to be acted on by sera known to agglutinate bloods of certain types. Such sera can be obtained from persons of groups A and B, respectively, provided that their serum has a good titer of agglutinin and gives rapid and strong agglutination. Stronger sera can be prepared from the serum of rabbits immunized with erythrocytes of groups A and B, then freed from nonspecific agglutinins by specific absorption (see page 423). The preparation of such absorbed sera requires considerable serological experience. Probably the best human blood grouping sera are prepared by injecting volunteers of group A and B with purified group A and B substance (126).

If enough blood is available, the results obtained by testing the unknown erythrocytes with known sera can be checked by testing the sera of the individuals of unknown groups against known cells. Reactions should be obtained indicating the presence of agglutinins in conformity with Landsteiner's rule (see page 169). For the most precise work these tests are both carried out simultaneously.

3. CROSS-MATCHING

Before carrying out a transfusion it is necessary, even if the donor is of the same group as the prospective recipient, to test whether the serum of the recipient agglutinates the cells of the donor, and vice versa (cross-matching). The test of the patient's serum against donor's cells is the more important, and should never be omitted.

Two sorts of tests, the blood group determination, and the direct (cross-matching) test, will usually be carried out. It is often asked which of these tests is to be preferred, but the question is incorrectly put. Each of the two tests has its special advantages and disadvantages, and a maximum of certainty is obtained if the two tests are carried out together. The importance of the diagnosis is such that it should be obvious that nothing which contributes to the certainty of the result should be omitted.

Institutions which often have to take care of very urgent cases, large hospitals, for example, should provide themselves with "blood banks" so as not to lose valuable time in the search for suitable donors (see 111).

For other cases, the "complete test" (111) gives the greatest possible certainty, and requires only about five to ten minutes to do. Persons of group O may be used in transfusions to patients of any blood group (so-called universal donor), if their blood is first "conditioned" by the addition of a sterile solution of Witebsky's A and B substances (127).

The "Complete Test"

If 1 or 2 cc. of blood are available from both donor and recipient, three tests, independent of each other, are set up: (*1*) The recipient's serum with the donor's corpuscles and (if convenient) the donor's serum with the recipient's cells; (*2*) Group determination of donor and recipient with known serum A (anti-B) and B (anti-A); and (*3*) Group determination of donor and recipient, with known corpuscles A and B.

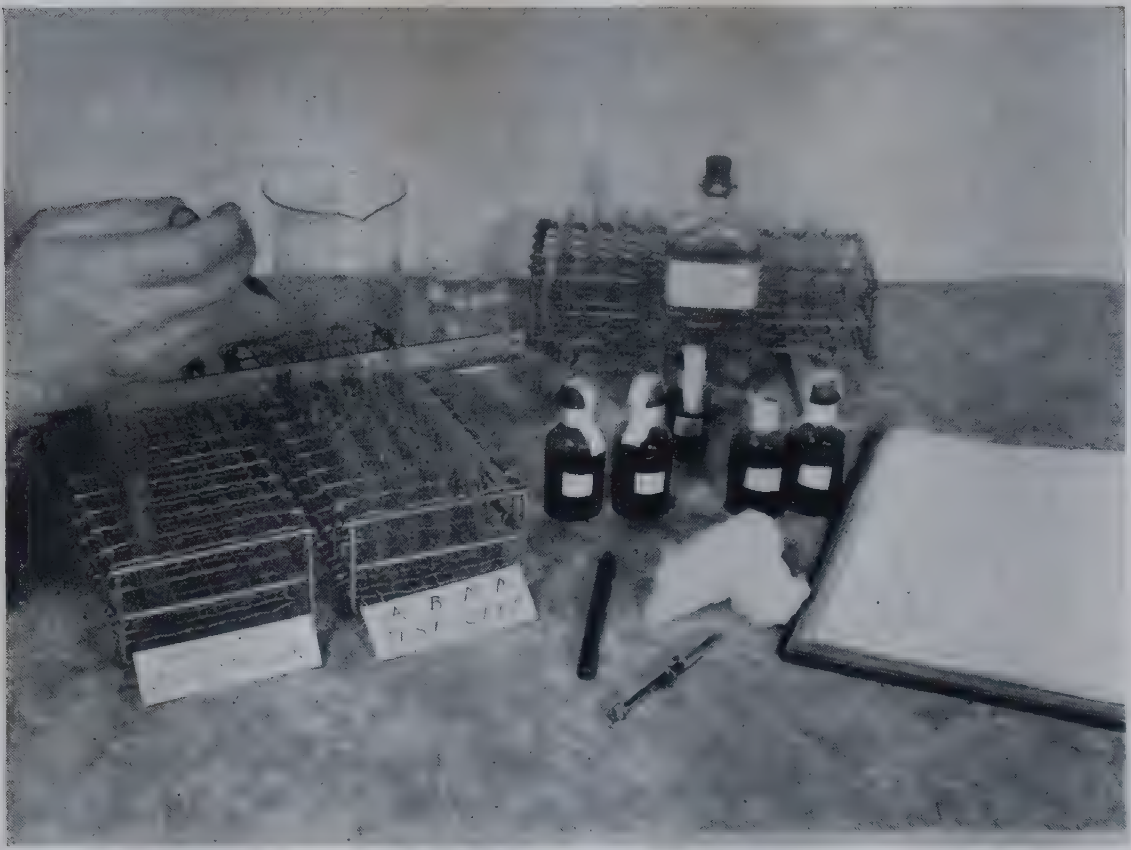

Fig. 44. Determination of blood groups and M, N types in test tubes (111).

Cell suspensions are prepared from the donor and the recipient by mixing a few drops of blood with saline. If the blood is in the form of a clot, and difficulties are experienced in pouring off cells, a glass rod or a pipet may be stuck into the clot and washed off into the salt solution until the desired density of suspension is obtained. The suspension is made up to about 1 to 2% cells, as judged by the eye. If it is too heavy, more diluting fluid may be added.

The tests are set up in test tubes (111). See Figure 44. If the usual serological apparatus (pipet, test tubes, centrifuge) is not available, the reaction is carried out on microscope slides. This must be done if test serum is available only in very small quantities. With very good reagents, such as those obtainable by Witebsky's method (see page 169), grouping on slides appears to be quite satisfactory, but cross-matching should if possible be done in test tubes.

The procedure of group determination with test serum A (anti-B) and B (anti-A) on the slide takes the following form: A drop of the blood to be investigated is added to physiological saline and evenly mixed by shaking or stirring. On the left of the slide there is placed a large drop of the test serum A (anti-B), and on the right or, as some prefer, in the middle of the slide, an equal drop of test serum B (anti-A). A large drop of cell suspension is added to each drop of serum. Complete mixing is secured first by stirring, later by rocking the slide to and fro. The progress of the reaction is observed against a white background. The test is observed for five, or at the most ten minutes. The diagnosis may be obtained from the adjoining scheme:

Serum A (anti-B), left-hand part of slide	Serum B (anti-A), right-hand part of slide	Indicated group
–	–	O
–	+	A
+	–	B
+	+	AB

On *repetition* of a transfusion the preliminary serological tests must each time be carried out anew, even when a donor already tested is being used again, for there is always the possibility that the previous transfusion may have produced *immune* antibodies against individual or group substances of the donor. Of the possible immune isoagglutinins which might conceivably be produced, only agglutinins against the Rh factor have thus far been shown to be very important (73, 125).

Technics adapted to mass grouping of large numbers of subjects in short periods of time have been described (95).

Tests of Standard Sera

The serologist must not be satisfied with having once checked his sera, but must convince himself of their suitability at intervals, depending on time elapsed, method of preservation, and sterility of the material. It is of course preferable to check the sera, not at the time a blood group determination is demanded, but at regular intervals (see 111).

Determination of the M, N Types

The M, N types of Landsteiner and Levine are determined in small test tubes (Fig. 44), making use of absorbed antisera specific for M and N (see page 423). Since this technic will not be required except by those especially interested in blood grouping or medicolegal problems, it will not be described here. It may be found in Wiener (124) or in Schiff and Boyd (111).

Agglutination Technic; Dilutions

In determining the titer (degree to which serum may be diluted) of sera for blood grouping, in agglutination experiments with antibacterial agglutinating sera, as well as in many other experiments, it is necessary to make serial dilutions of the serum or some other reagent. Such dilutions may be conveniently made in "Wassermann" tubes (page 397). The steps may be small, e.g., each dilution double the preceding, or large, e.g., tenfold, depending on the strength of the serum and the purpose of the experiment.

Titration of Blood Grouping Sera

In titrating blood grouping sera, successively doubled dilutions are usual. In each of as many tubes as desired (12 for example), place 0.5 cc. of saline. This may be measured out rapidly with a 2-cc. graduated pipet, filling four tubes at a time. In the first tube place, with a 1-cc. serological pipet, 0.5 cc. of the serum, and mix by drawing up most of the liquid into the pipet and expelling it, repeatedly. This mixing should not be so vigorous as to produce foam, for foaming may denature proteins in solution. This gives a 1:2 dilution. Then with the same* pipet transfer 0.5 cc. of the mixture to the next tube, mix, and continue in this way until all the dilutions are made. Discard 0.5 cc. of the mixture in the last tube. If it is not certain that negative readings will be obtained with the last few tubes, a tube containing 0.5 cc. of saline should be included as a control.

To each tube is now added a measured amount of the cell suspension, the amount depending on the strength of the suspension. The amount should be adequate to give a definite pink color to the mixture, but not too great, as agglutination will be slower with concentrated suspensions. Much time is saved if the centrifuge is used to hasten the agglutination, unless too many tests have to be set up at once. If the centrifuge is to be used, it is preferable to use smaller volumes. As the dilutions are being made, transfer 0.2 cc. of each to one of the smaller test tubes recommended for this purpose above. Then add to each tube 0.1 cc. of the erythrocyte suspension (about 1% in strength). Centrifuge at about 1200 rpm for a constant time (e.g., three minutes). Replace the tubes in a rack in which they do not fit too tightly and shake the entire rack until the contents of the control tube are thoroughly suspended.

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* Some accuracy is lost by using the same pipet, with its adhering solution inside and outside, but such a procedure is traditional; it is more convenient, economical of time and glassware, and accurate enough for most purposes.

4. BACTERIAL AGGLUTINATION

It is customary to carry out bacterial agglutinations in "Wassermann" tubes, with quantities of reagents sufficient to give a final volume of 1 cc. The bacterial suspension may be prepared from a broth culture or a saline suspension of bacteria grown on solid media. We may give the technic of the Widal test (page 387) as an example.

The Widal Test. Take about 3–5 cc. of blood from the patient, under aseptic conditions, and place it to clot in a sterile, stoppered (cotton-plugged) tube. Remove the serum and centrifuge to eliminate erythrocytes. Remember that the material may be dangerously infective. Using sterile pipets (see page 398) dilute 0.2 cc. of the serum with 4.8 cc. of sterile saline to obtain a 1:25 dilution of the serum. Place 0.5 cc. in an empty sterile test tube, and 0.5 cc. in a tube containing 0.5 cc. of sterile saline, giving a dilution of 1:50. Then proceed, as described on page 414, to make successively doubled dilutions of 1:100, 1:200, 1:400, 1:800, leaving 0.5 cc. in each tube. Including the saline control, this gives a row of seven tubes. Prepare six such rows in all.

The bacterial suspensions used are rather light (practically transparent), of a density equal to tubes five or six of McFarland's nephelometer (69). They should be free from clumps and those for the "H" agglutination are preserved by the addition of formalin to 0.2%. The "O" suspensions are prepared by making thick suspensions of the bacteria, placing them in containers (graduated cylinders for example), and adding an equal volume of ethyl alcohol while stirring. The cylinder is covered and kept at 37°C. for 12–24 hours. The flocculent precipitate which forms is resuspended and the material kept at room temperature for two to three hours, then an equal volume of saline is added. This stock solution keeps indefinitely. The original suspension should be thick enough to allow the final mixture to be diluted at least 1:6 before use, in order that the alcohol content should be reduced sufficiently in the dilution made before actual use.

To all the tubes of each row there is added 0.5 cc. of one of the following suspensions: typhoid "H" (see page 155), para A "H", para B "H", typhoid "O", para A "O", and para B "O". The final dilutions of serum have been doubled by this addition, and now are 1:50, 1:100, etc. Shake well, incubate in a water bath at 52°C. for two to four hours (four hours for the "O" agglutinin). Place in the ice box overnight and read the following morning. The sediment of bacteria in the control tube will be resuspended easily by gentle shaking. Positive "H" agglutination consists of fluffy, cotton-like clumps at the bottom of the tube, while typical "O" agglutination gives a finely granular precipitate of agglutinated bacteria (2).

The test should be made, for both H and O agglutinins, with all the varieties of the *Salmonella* group likely to be of local importance at the time (35). Considering any one organism, the typhoid bacillus for example, we have four possibilities (104), which may be summarized rather roughly thus: (*a*) Both H and O agglutinations are negative, in which case the disease is probably not typhoid (and probably not any other member of the group), or else the infection is too new for agglutinins to have developed. (*b*) The O may be positive and the H negative. This means the disease is not typhoid, or if typhoid, too recent for the H agglutinins to have developed. If not typhoid, the disease is some other of the group. (*c*) The O may be negative and the H positive. This means the disease is not now active, or the patient has previously been vaccinated against typhoid. The O agglutinins arise more quickly during infection, but are also lost more quickly. (*d*) Both O and H may be positive. This indicates typical typhoid which has run long enough to produce both agglutinins (about one week for O and about two to three weeks for H).

The diagnosis of typhoid is fairly certain, in the case of a person not previously immunized, only if a high titer of H agglutinins, or an O titer well above normal is found, and in the case of persons not too recently immunized, only if a high H titer, or an O well above normal, is found. We may provisionally define an H titer as high if it goes to 1:250, and very high if it goes to 1:1000, and an O well above normal if it goes to 1:250, but these can not be regarded as rigid definitions, depending rather on the observed level in the normal population, and the vaccine with which the patient was immunized, and how long ago (117).

The Widal test is also sometimes carried out by the *microscopic method* in a hanging-drop preparation. This is less reliable, but has the advantages of enabling the reactions to be read more quickly, and of taking less serum. The technic is described by Kolmer (69).

Bacterial agglutination reactions are also sometimes done on a slide, with direct instead of microscopic observation. A portion of a bacterial colony (the remainder may be smeared, stained, and checked for type of organism and purity of culture) is emulsified in two drops of saline, on a clean slide, and a loopful is placed at the opposite end of the slide to serve as a control. A drop of serum is mixed with the original drop, the slide is rocked to secure mixing, and the reaction is observed against a black background. If the reaction is positive, granules appear in the mixture, while the control drop remains homogeneous (2). This reaction is better adapted to the identification of unknown organisms with known immune agglutinating serum than to performance of the Widal test. The *Bass-Watkins* test is a rapid method of determining the presence of antityphoid agglutinins in small quantities of dried blood.

A rapid slide test ("Welch test") for the diagnosis of typhoid, paratyphoid, and typhus fevers and brucellosis has come into favor in many laboratories (62, 123). In addition to its rapidity, the test has the advantage that the antigens are more stable (good for at least six months), and can be purchased.

The serum to be tested is pipetted in amounts of 0.08, 0.04, 0.02, 0.01 and 0.005 cc. with a pipet holding 0.2 cc., graduated in thousandths, into depressions or wax rings on a glass plate. The glass plates are made up to have six rows of five rings each, and each row receives the above doses of serum. A drop (0.03 cc.) of typhoid "O" antigen is added to each of the serum amounts in the first row. Similarly, typhoid "H" antigen is added to each ring in the second row. Paratyphoid "A" is added to the third row, Paratyphoid "B" to the fourth, *Proteus OX 19* to the fifth, and *Brucella abortus* to the sixth. Each row is stirred thoroughly with a clean instrument such as a toothpick, starting with the smallest amount of serum and working from right to left. Positive and negative controls should be set up with the first of each day's tests. The slide is rocked gently to and fro 20–30 times, and the degree of agglutination after each five separate rockings is checked.

The results are interpreted as is the test tube reaction, remembering that the amounts of serum used give, after addition of the antigen, dilutions corresponding to 1:20, 1:40, 1:80, 1:160, 1:320 in the test tube test. In cases in which the patient has never received vaccine and has the proper clinical symptoms positive agglutination to a titer of 1:80 may be considered significant.

Dried bacteria have been used in Germany for the preparation of suspensions for certain sorts of diagnostic tests (115).

5. Pneumococcus Typing

The Neufeld technic has practically replaced all others in this procedure. It is based upon the swelling of the capsule of the organisms when treated with antisera of the corresponding type.

Place a small fleck of sputum on a clean cover slip by means of a small platinum loop. With a large cool platinum loop, add four to five times as much typing serum as sputum; mix thoroughly. Invert the cover slip on a glass slide, and press it down to make the layer between the cover slip and slide thin and even. Let stand about five minutes, then examine under the oil immersion lens.

The typing sera have blue dyes added to them to facilitate reading, so that in a positive reaction dark blue diplococci surrounded by an unstained area with definite outline are seen. In a negative reaction the capsule does not swell, and the unstained surrounding area is either not seen or is narrow (Fig. 45). Sharpness of outline is an important feature of the positive reaction, so that the stain should always be used.

Since there are now over 70 types of pneumococci, carrying out the test *seriatum* with antisera for each type would be impracticable. It is customary to combine types of serum into a limited number (e.g., six) of mixtures. The test is then carried out with each mixture, and if a positive reaction is obtained with any one mixture, the typing is then completed by tests with each of the monovalent antisera contained in the mixture which reacted. Complete typing should always be carried out. If no capsular swelling is obtained in any of the mixtures, they should be allowed to stand 30–60 minutes in a moist chamber at room temperature before a final reading is made. More than one type of pneumococcus may be found. In addition, cross reactions within the types occasionally occur. The latter can be eliminated by diluting the sera 1:10 or 1:20 and repeating the typing. Unclassified types may sometimes be found.

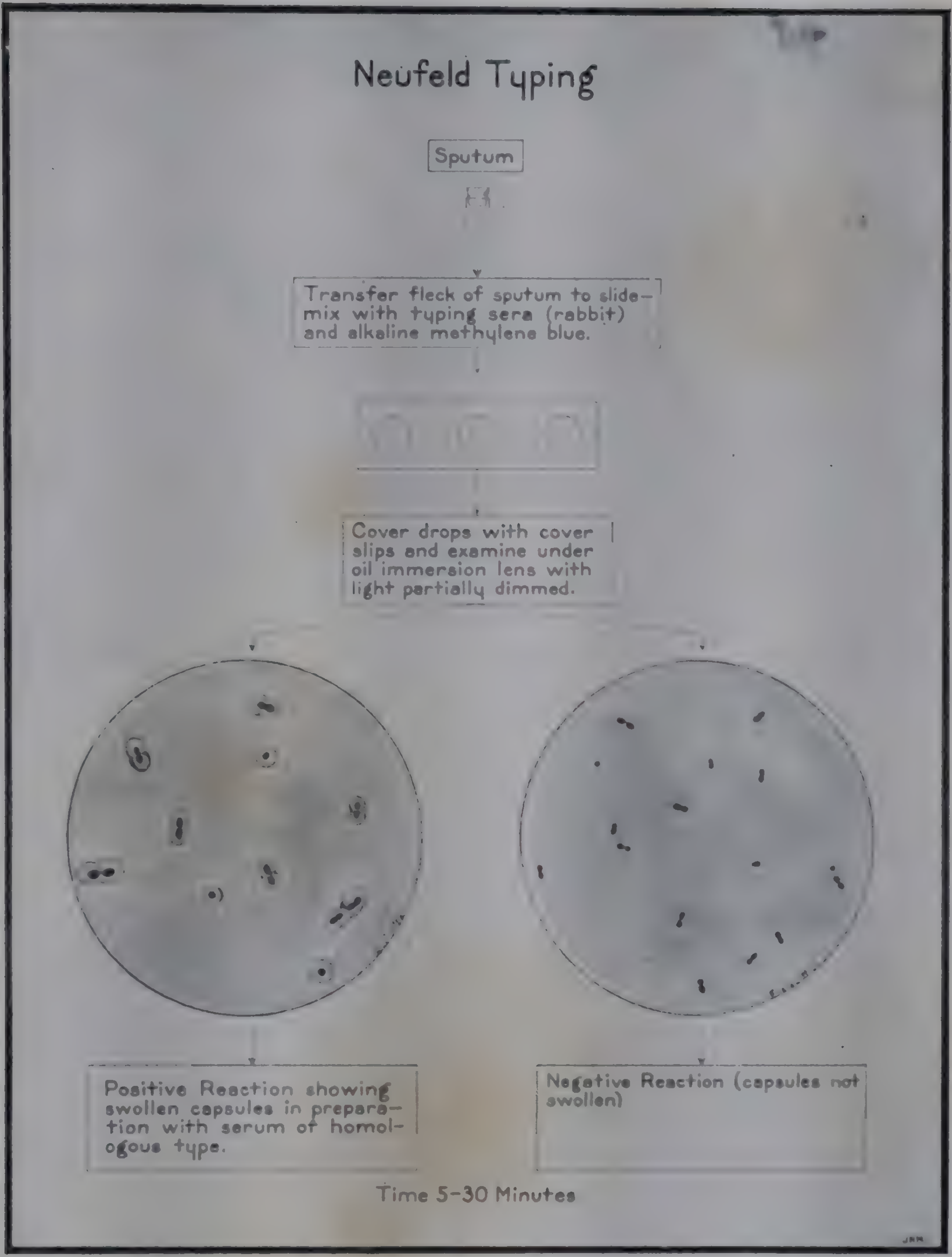
Neufeld Typing
Sputum
Transfer fleck of sputum to slide—mix with typing sera (rabbit) and alkaline methylene blue.
Cover drops with cover slips and examine under oil immersion lens with light partially dimmed.
Positive Reaction showing swollen capsules in preparation with serum of homologous type.
Negative Reaction (capsules not swollen)
Time 5-30 Minutes

Fig. 45. Schematic representation of the Neufeld method of typing pneumococci *(courtesy of the Massachusetts Department of Public Health)*.

The sputum used should be coughed up from the lungs, and should be examined while fresh, preferably not over an hour old. Sometimes sputum which has been kept on ice a day or so can be typed. If the test is inconclusive, or very few pneumococci are found, a portion of the sputum may be inoculated into Avery's medium and the organisms which have grown after incubation typed (2).

"Mouse Typing." This earlier "classical" method of typing pneumococci is satisfactory but takes much longer. An emulsion of some of the washed sputum is injected into the abdominal cavity of a mouse. An attempt may be made to obtain exudate from the abdominal cavity, using a sterile needle and syringe, at the end of three to five hours, or when the mouse appears sick. If no exudate is obtained, the mouse is allowed to live 18–24 hours, unless it dies before this time. Then it is killed and organisms recovered from the heart for culture and for agglutination tests for typing. Centrifuged saline washings containing the soluble specific substance from the abdominal cavity can be tested for type by the precipitin reaction, using the same sera as above. The mouse method is still used when direct typing is not possible because of the small number of organisms in the sputum. Typing from the peritoneal exudate is easily done by the Neufeld technic.

6. Technic of the Precipitin Reaction

A precipitating serum, if mixed with the homologous* antigen in the proper proportions, will produce a clouding of the originally clear fluids, usually followed by the formation of a flocculent precipitate. The reaction is more easily seen, and takes place over a wider range of concentrations, if it is carried out by placing one fluid (the antigen) over the other (the antiserum) in a suitable test tube. By this *interfacial technic* the reaction is visible as the formation of a white zone or plane at the junction of the two clear fluids. This is often called the *"ring" test.*

Reagents

The reagents involved are two: antiserum and antigen. The preparation of both has been described above. Both should be crystal clear (though not necessarily colorless). If not clear, they should be filtered through a retentive filter paper, and if necessary centrifuged a long time at high speed. The antiserum is more likely to be clear if the animal is fasting when bled; it is used undiluted, or sometimes diluted 1:2 or 1:4. A number of different dilutions of the antigen should be tested. If successive dilutions 1:10 are made of the stock antigen, giving dilutions of 1:10, 1:100, 1:1000, etc., six dilutions will be sufficient.

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* The homologous antigen is antigen from the same source, i.e. the same species, as that used in injecting the animal furnishing the antiserum.

Apparatus

It is convenient to make the antigen dilutions in test tubes about 13 × 100 mm. ("Wassermann tubes"), using ordinary serological pipets. The reaction may be carried out in test tubes of 9 × 110 mm., putting in 0.9 cc. of antigen dilution, and 0.1 cc. of antiserum (Uhlenhuth). Much material may be saved, and the reaction rendered easier to read, by using "micro" tubes made from semicapillary tubing, about 35 mm. long and 2.5 mm. internal diameter. In these there is placed about 0.04 cc. of antiserum and on top of this about 0.04 cc. of antigen dilution.

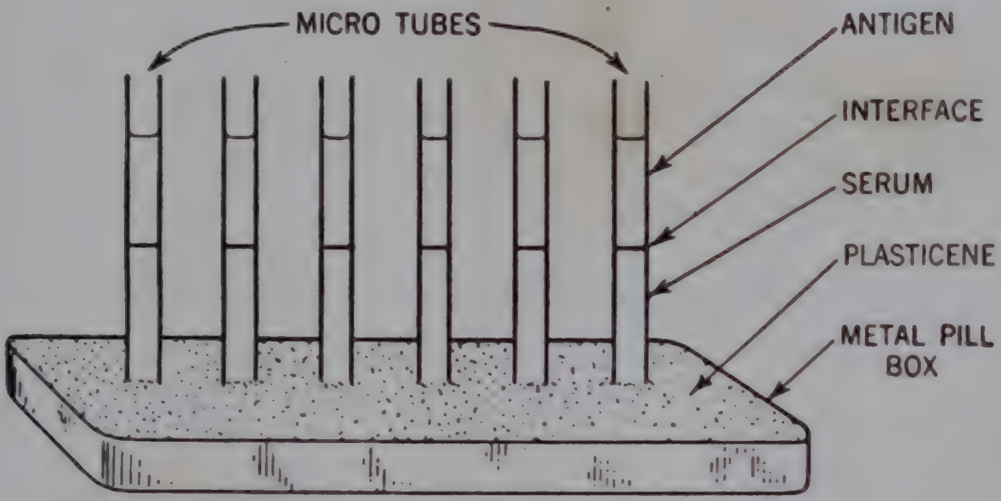

Fig. 46. Set-up for the precipitin test for human blood (111).

The "micro" tubes may be supported by sticking their lower ends into plasticene in a small shallow container, such as the metal cover of a pill box (Fig. 46). Or special small racks of wire may be constructed, which have the advantage of leaving the bottom of the tube open to inspection, and of not soiling the outside of the tube.

For use with the small tubes, fine capillary pipets, made by drawing ordinary laboratory glass tubing to a point in a flame, and small rubber bulbs (two-cc. capacity) will be needed. With a little practice, it is easy to transfer with these the desired amount of fluid into the little precipitin tubes and obtain the desired sharp line of junction.

Determination of Titer of Antiserum

It seems sufficient in this work to estimate the strength of the antiserum by determining the highest dilution of antigen which is precipitated by the antiserum. This is particularly appropriate here as we are interested in having a serum which will detect animal proteins, if necessary in very low concentration. The strength of the serum may be roughly estimated by the amount of precipitate produced when the contents of the tubes are mixed and allowed to stand overnight.

The antiserum is tested with successive dilutions, for example, 1:10, 1:100, 1:1000, etc., of the homologous antigen (e.g., human serum), and the last dilution noted which gives a visible reaction in 20 minutes or less. This is the titer of the antiserum. If desired, dilutions in smaller steps, such as 1:5, 1:25, . . . or 1:3, 1:9 . . . , may be tested.

A serum with a titer of 1:20,000 or over is satisfactory. However, the amount and density of precipitate, and the rapidity of the reaction, are also of significance.

The tests are set up by transferring about 0.04 cc. of antiserum to each of six or seven of the small tubes described above, using the capillary pipet and rubber bulb. Serum in one of the tubes is overlayered with saline, for the control. Then with another capillary pipet, or with the same pipet *thoroughly* rinsed, there is placed over the serum and in contact with it so as to form a sharp junction without bubbles, about 0.04 cc. of each of the antigen dilutions in the other tubes, keeping them in order in the support, or marking them to prevent confusion. Begin with the most dilute solution and progress to the most concentrated, without rinsing. A control consisting of the strongest concentration of antigen overlayered with saline is also desirable.

Characteristics of a Positive Reaction

If the above technic is used, a positive reaction is seen in the form of a thin white ring, or rather, plane, at or very near the junction of the antiserum and antigen, which appears within a few minutes after the antigen has been added, and grows heavier. In a very strong reaction, particles of precipitate may begin to fall down through the antiserum. The disk of precipitate should remain horizontal if the tube is tipped, showing that it is not dirt or other contamination attached to the walls of the tube.

The reactions should be read in a good light, with a dark background behind the tubes. A well-lighted window which has a cross bar which can be used for the background is very satisfactory. Fluorescent illumination with a dead black background is good.

Forensic Precipitin Tests

Before attempting to determine the species origin of a stain or other specimen of dried blood, it is necessary to ascertain first of all that it is blood (benzidine or hemochromogen test), then to determine by the precipitin test if it is human blood.

Production of Antiserum

Precipitins suitable for differentiating human from other blood may be produced by injecting laked whole human blood rendered again isotonic by the addition of salt, or by injecting defibrinated whole blood, plasma, or serum. Serum is easiest to procure, and seems on the whole as satisfactory as anything else.

It may be well to dilute the serum before injecting it to an approximately 1% protein content (i.e., dilute 1:6 or 1:7). This is easiest before sterilization (if this is required) and before putting up in bottles for injection. Such solutions seem to be strong enough, and injection of them is borne well by the animals. The (diluted) serum is conveniently kept in sterile bottles fitted with rubber stoppers having a thin spot to allow introduction of a sterile hypodermic needle to withdraw material.

The tests are carried out as described immediately above. Reactions are to be judged positive when they fulfill the conditions laid down there.

Test of Specificity of Antiserum

Before use in practice, each antiserum must be tested for specificity. If it is to be used in America or Europe in the usual type of medicolegal case, it will probably be sufficient to ascertain that it does not react with any of a series of dilutions of the blood of common domestic animals: horse, dog, sheep, pig, cat, chicken, goose, etc. If it is desired to differentiate between human blood and the blood of anthropoid apes and monkeys, it will be necessary to test the antiserum with these bloods also. It is probable that a reaction will be found with the bloods of the higher apes. In some cases also a reaction will be obtained with (low dilutions only) various mammalian bloods (mammalian reaction of Nuttall, 94). Some antisera may react weakly with human milk.

Production of a Completely Specific Antiserum

In case the antiserum is found to react with any of the nonhuman samples tested, it may be rendered specific by absorption. That is, some of the reactive heterologous antigen is added, the precipitate is removed, and the absorbed antiserum is thus deprived of its power to react with this heterologous material.

The procedure of specific absorption is described on page 423.

Preparation of the Unknown Specimen for Test

Before the reaction can be carried out, the unknown material must be brought into solution. If the blood stain is on hard material such as glass, smooth wood, and metal, it should be scraped off, powdered, and the powder extracted. A roughly equal amount of scrapings from the unstained substrate should be extracted in the same way for use as a control. If the stain is on cloth, paper, etc., the stain, or a portion of it if it is large, should be cut out and extracted. An equal portion of unstained material should be extracted at the same time.

The material is extracted with physiological saline (0.9% sodium chloride solution), overnight in the ice box. It is suitable to use about one cubic centimeter of saline (more if the material is very absorptive) for each square centimeter of stained material, in the case of cloth, etc., or for each four to five milligrams of dried blood powder. This should give a solution, if all the dried serum proteins dissolve, roughly equivalent to a 1:10 or 1:20 dilution of fresh serum. The actual content of dissolved protein should be estimated, simultaneously with the precipitin test, by shaking portions of the various dilutions to produce foam, and by placing small amounts of them over concentrated nitric acid in little tubes, and noting the greatest dilution of the extract which gives a reaction; this will correspond to a dilution of about 1:1000 of fresh serum. The dilution which just forms stable foam is similar (Uhlenhuth).

The extract should be filtered and if necessary centrifuged at high speed to clarify it.

Setting Up the Precipitin Test Itself

Over the serum will be placed, in five of the antiserum-containing tubes, a 1:100,000, a 1:10,000, a 1:1000, a 1:100, and a 1:10, respectively, dilution of the extract of the unknown stain. For this the same pipet may be used, beginning with the most dilute solution and progressing to the most concentrated, without rinsing. When the 1:100 dilution is reached, some should also be placed over normal rabbit serum. Then with separate pipets, saline is placed in another antiserum-containing tube, and an extract of the unstained substrate in another, in that order. With another pipet a 1:1000 dilution of some heterologous blood serum* is added to an antiserum-containing tube, and a 1:1000 dilution of known human serum is placed in another tube also containing antiserum. The set-up will have the appearance shown in Figure 47.

Purpose of the Controls

In order to be able to make positively the diagnosis of human origin of unknown material, the rather elaborate controls outlined are needed. *Normal rabbit serum* (tube 6) used to test if the extract of the unknown material will give a (nonspecific) precipitate with sera not containing antihuman precipitins. Extracts of some materials have a nonspecific precipitating power when mixed with sera. The reaction in this tube must be negative. *Saline* (tube 7); this tests if the antiserum tends in general to cloud when in contact with other, nonspecific fluids. This tube also serves as a standard with which to compare the appearance of the other reactions. The reaction here must be negative. *Substrate* (tube 8); this tests if the substrate has, independently of the stain, any specific or nonspecific precipitating power. The result here must be negative. *Heterologous blood* (tube 9); this controls the specificity of the antiserum, and shows that it does not react with mammalian bloods in general†—at least not in high dilution. The reaction here must be negative. *Known human blood* (tube 10); this controls the activity of the antiserum. It is necessary to show that the serum still reacts well. This tube also serves as a standard reaction with which to compare any positive reaction found with the unknown.

These controls are of great importance; no one of them should be omitted.

Evaluation of the Results

If the controls are satisfactory (tubes 6–9 negative, tube 10 positive), positive reactions appearing in tubes 1, 2, 3, or 4 indicate that the blood stain was of human (or anthropoid) origin. A reaction in

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* If there is any clue to the possible origin of the stain, assuming that it is possible that it is not human, blood of the suspected species should be used here.

† In case the results are to be presented in court, the protocols may be more impressive if several heterologous bloods are tested.

Scheme for the Complete Precipitin Test, with Controls

	Tube number									
	Tests					Controls				
	1	2	3	4	5	6	7	8	9	10
Upper layer	Extract, 1:100,000	Extract, 1:10,000	Extract, 1:1,000	Extract, 1:100	Extract, 1:10	Extract, 1:100	0.9% NaCl	Substrate extract	Heterologous blood, 1:1,000	Known human blood, 1:1,000
Lower layer	Antiserum	Antiserum	Antiserum	Antiserum	Antiserum	Normal rabbit serum	Antiserum	Antiserum	Antiserum	Antiserum
Expected result						—	—	—	—	+

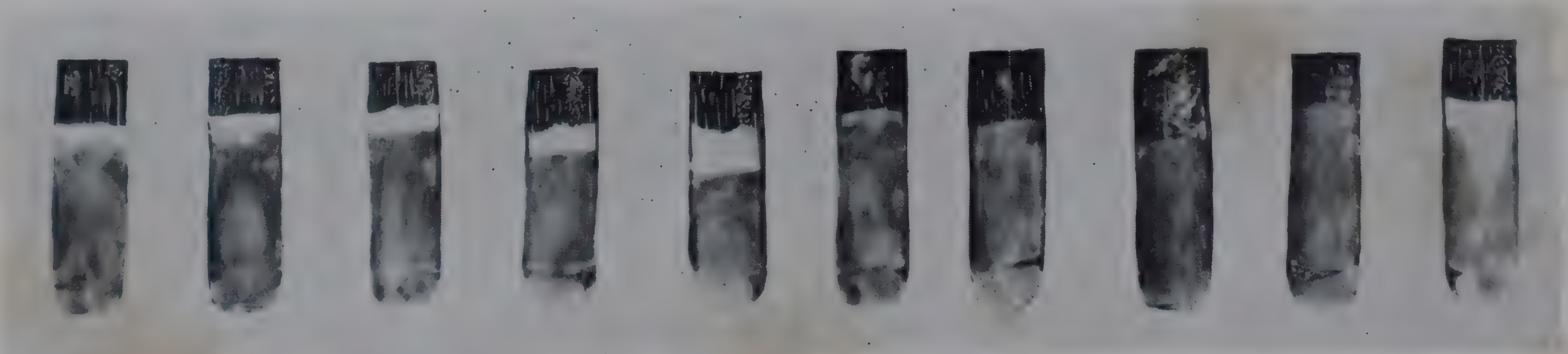

Fig. 47. Photograph of precipitin tests. Counting from the left, a weak positive reaction is seen at the interface between serum and antigen in tube 1. Tubes 2, 3, 4, and 5 are positive. The controls are negative, or positive, as required (15).

tube 5 alone is of less significance,* unless the nitric acid test shows the original extract to have been unusually weak. A reaction in tube 4 alone is probably significant, but shows the concentration of serum proteins to be very low in the extract. In a clear cut case, positive reactions will be obtained in tubes 5, 4, 3, and possibly 2 and 1.

Limits of Applicability of Test

A positive reaction, as described, with satisfactory controls, is diagnostic for human (or ape) blood. Human blood can even be differentiated from anthropoid blood by using a good antiserum, specifically absorbed. Human blood stains 15–60 years old have been identified by the precipitin test.

A verdict in the opposite sense, i.e., that a stain is *not* human blood, should be ventured only if the stain is in a good state of preservation, not too old, and preferably only if a good reaction can be obtained on testing it with an antiserum to blood of some animal species, thus positively identifying it.

In case the extract of the unstained substrate causes a clouding, a confident diagnosis will be impossible. Sometimes neutralizing the extract, (i.e., bringing it to pH 7) may remedy this.

The forensic use of the precipitin test for human blood has been discussed from the point of view of the layman and the legal profession in a recent article (15).

A "thermoprecipitin" test for anthrax (Ascoli, 1a, 1b), is important. It enables infected hides, furs, etc. to be detected. It evidently depends largely on a reaction with the somatic carbohydrate of the anthrax bacillus (71).

7. Complement Fixation

This forms part of a number of diagnostic and experimental procedures. Its most frequent application is as part of the Wassermann reaction, therefore the technic will not be described separately. It may be found in the next few paragraphs describing a Wassermann test.

8. Wassermann Reaction

The theoretical bases of the Wassermann reaction have been discussed on page 302. There have been many modifications of the original technic of this reaction, and it would be impossible and pointless to describe them all. Therefore the reaction as carried out in the author's own institution will be described, although it is not necessarily typical of all.

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* Particularly if the antiserum used is known to precipitate low dilutions of certain heterologous bloods.

Reagents

Saline. Evidently 0.9% sodium chloride solution is closer to being isotonic with blood serum than is 0.85% (54). However, according to Eagle (29) 0.85% saline is best for the Wassermann test. Many workers find the results of hemolysin titrations more reproducible if to each liter of this one gram of magnesium sulfate is added (2).

Sheep Cells. Blood is taken from the sheep into citrate, or the blood is defibrinated, and the cells centrifuged down. About ten cubic centimeters of cells (depending on the number of tests required) are placed in a 50-cc. centrifuge tube which has been rinsed with saline; the cells are mixed with about 30 cc. of saline and centrifuged down, and the supernatant is removed and discarded. The washing is repeated two or three times. After the last centrifugation, which should always be at the same speed and for the same time, the volume of the packed cells is recorded.

Anti-sheep Hemolysin. This is an immune serum prepared by injecting rabbits with a 30% suspension of sheep erythrocytes. One of the injection schedules mentioned on page 402 may be followed. The lytic titer should be as high as 1:4000. The serum can be sterilized by Berkefeld filtration, or preserved by mixture with an equal volume of glycerine, or by Merthiolate. The lysin is sometimes referred to as amboceptor, or sensitizer.

Complement (Alexin). Guinea pig serum is the best source. Take blood by cardiac puncture, or by cutting the jugular vein of the anesthetized animal; pool the blood from four or more pigs, put it into flat dishes and allow to stand one hour at room temperature. Then loosen the clot around the edge and put the dishes on ice over night. Next morning pour off the serum, centrifuge out the erythrocytes, and preserve what is not required for the day's work. A number of methods have been suggested for preserving complement (see 29). The simplest is probably "salting" (the addition of 0.085 g. of dry salt for each cubic centimeter of serum). Such salted complement keeps in the ice box for five to seven days, declining in activity the while. Before use it is diluted 1:10 with water. Much more satisfactory is drying the complement in the lyophilizing apparatus of

Flosdorf and Mudd, which seems to preserve it perfectly for long periods of time. A large lot of pooled complement may be prepared, dried in small lots, and water added to a lot when complement is needed. Such dried complement is also available commercially.

Antigen. The antigen consists of an alcoholic solution of lipoid substances from normal mammalian tissue, usually beef hearts. Any number of methods of preparing it have been proposed. According to Eagle (29) the preferable method is to extract the dried tissue first with several volumes of ether, then with alcohol. Sterols (e.g., cholesterol) are added to increase the sensitivity. The antigen must not be hemolytic, must not be anticomplementary (bind complement alone) except in large amounts, and must combine with syphilitic reagin in small amounts. These points must all be checked when a new lot of antigen is prepared. Methods of preparation are described by Kolmer (69) and Wadsworth (122). Antigens are also available commercially.

Preparation for the Test

Blood (five cubic centimeters) is taken from the patient by venipuncture with a dry syringe and put in a dry tube to clot. If necessary the clot is loosened with a wooden or glass rod, and centrifuged. The serum is removed with a clean dry capillary pipet and heated in a water bath at 56°C. for 20 minutes to destroy any complement present, and to lessen the serum's anticomplementary property. When sera are to be retested they should be reinactivated.

Make a ten per cent suspension of the washed sheep cells. For example, if the volume of cells is 4.5 cc., add enough saline to make the final volume 45 cc. Keep the suspension on ice until ready to use, and mix well immediately before using.

The amount of lysin to use is determined by preliminary titration (Table XLIX). The titrated lysin is diluted so that a given volume contains enough lysin (two units) for an equal volume of ten per cent sheep cell suspension. Mix the required amounts of these two reagents by pouring back and forth from one beaker to another. Keep on ice until needed.

A 1:20 dilution of guinea pig serum may be used for preliminary titrations. If salted complement is used, dilute first 1:10 with water, and dilute this 1:2 with saline. The scheme of titration is shown in Table L.

Dilute the stock antigen by putting the required amount into a clean beaker rinsed with saline and adding the requisite amount of saline drop by drop at first, more rapidly later. An opalescent mixture will be obtained. The optimal dilution of the stock is determined by experiments with varying dilutions of stock and known positive serum (Table LI-A). (See Hooker, 55, and Eagle, 29.) Some

Table XLIX

Titration of Hemolysin

Tube No.	Lysin (anti-sheep serum 1:500[a]), cc.	Saline, cc.	Complement (guinea pig serum 1:20), cc.	Cells (5% sheep cells), cc.
1	0.20	0.45	0.25	0.10
2	0.16	0.49	0.25	0.10
3	0.13	0.52	0.25	0.10
4	0.10	0.55	0.25	0.10
5	0.08	0.57	0.25	0.10
6	0.06	0.59	0.25	0.10
7	0.04	0.61	0.25	0.10
8	0.03	0.62	0.25	0.10
9	0.02	0.63	0.25	0.10
10 (lysin control)	0.20	0.70	—	0.10
11 (complement control)	—	0.65	0.25	0.10
12 (cell control)	—	0.90	—	0.10

Water bath at 37°C. for 30 minutes

The unit of lysin is the smallest amount of lysin producing complete lysis of test dose of cells (0.10 cc.) in the presence of an excess of complement. Note that the amounts of saline added are such as to give always a volume of 1.00 cc.; this is because concentration is a factor in the activity of complement.

[a] This dilution chosen by preliminary rough test.

Table L

Titration of Complement

Tube No.	Complement (guinea pig serum 1:20), cc.	Saline, cc.	Lysin (1 unit per 0.1 cc.), cc.	Cells (cc.)
1	0.20	0.50	0.2	0.1
2	0.17	0.53	0.2	0.1
3	0.14	0.56	0.2	0.1
4	0.12	0.58	0.2	0.1
5	0.10	0.60	0.2	0.1
6	0.08	0.62	0.2	0.1
7	0.06	0.64	0.2	0.1

Water bath at 37°C. for 30 minutes

The unit of complement is the smallest amount of complement causing complete lysis of the test dose of cells sensitized with two units of lysin.

workers attempt to determine the "antigenic titer" of an antigen by determining the quantity which is just sufficient to react with a known positive serum (for example, 2). According to Hooker (55) and Eagle (29), such determinations have no absolute significance, for the value will depend very much on the reagin content of the serum used. The optimal dilution furnishes all the information needed, and by determining it we avoid this false appearance of exact quantitation. It may be determined once for each lot of antigen, and need not be repeated each day, as the antigen titer is in many laboratories.

TABLE LI

TITRATION OF WASSERMANN ANTIGEN

(A) Determination of Optimal Dilution of Stock Antigen

(α) Amounts of Reagents

	Antigen dilution (see below)	Known positive serum dilution (see below)	Complement (1 unit in 0.25 cc.)	Water bath	Sensitized cells
Each tube	0.1 cc.	0.2 cc.	0.5 cc.	37°C. 60 min.	0.2 cc.

Water bath at 37°C. for thirty minutes

The optimal dilution of the stock antigen is that which gives fixation with the highest dilutions of known positive serum.

(β) Dilutions of Antigen Stock and Known Positive Serum Used in Above

Antigen dilution	Positive serum dilutions							
	Straight	1:2	1:4	1:8	1:16	1:32	1:64	1:128
1:10	+	+	+	+	+	0	0	0
1:20	+	+	+	+	+	±	0	0
1:40	+	+	+	+	+	+	±	0
1:80	+	+	+	+	+	+	+	±
1:160	+	+	+	+	+	+	+	±
1:320	+	+	+	+	+	+	±	0
1:640	+	+	+	+	+	±	0	0
1:1280	+	+	+	±	±	0	0	0

The signs indicate the degree of fixation as in the usual Wassermann test. + means positive, ± doubtful, and 0 negative. In the above hypothetical case the results show the optimal dilution of the stock antigen is between 1:80 and 1:160. The experiment is now repeated with intermediate dilutions, such as 1:80, 1:100, 1:120, 1:140, and 1:160, and the final decision is based on these results.

The hemolytic and anticomplementary titers of that dilution of

TABLE LI (cont.)

(B) Hemolytic Titer

Tube No.	Antigen (opt. dil. see above), cc.	Saline	Complement (1 unit per 0.15 cc.), cc.	Cells, cc.
1	0.6	—	0.3	0.1
2	0.4	0.2	0.3	0.1
3	0.3	0.3	0.3	0.1
4	0.2	0.4	0.3	0.1
5	0.1	0.5	0.3	0.1
6	0.05	0.55	0.3	0.1
7 (complement control)	—	0.6	0.3	0.1
8 (cell control or saline control)	—	0.9	—	0.1

Water bath at 37°C. for 30 minutes

The hemolytic unit is the smallest amount of antigen just beginning to produce hemolysis.

(C) Anticomplementary Titer

Tube No.	Antigen (as in B), cc.	Saline	Complement (1 unit per 0.15 cc.), cc.	Water bath	Sensitized cells, cc.
1	0.5	—	0.3	37°C. for 60 min.	0.2
2	0.4	0.1	0.3		0.2
3	0.3	0.2	0.3		0.2
4	0.2	0.3	0.3		0.2
5	0.1	0.4	0.3		0.2
6	0.05	0.45	0.3		0.2
7 (antigen control)	—	0.5	0.3		0.2

Water bath at 37°C. for 30 minutes

The anticomplementary unit is the smallest amount of antigen producing some inhibition of hemolysis.

the stock which is found to be optimal are next determined (Tables LI-B and LI-C). These tests are to be carried out exactly as the complete test will be later. The hemolytic activity of antigens prepared by modern methods is generally so slight that it can be ignored. The antigen should never be used in a concentration over one-fourth of that which is found to be anticomplementary.

Schemes for the series of titrations needed are shown in Tables LI-A, LI-B, and LI-C.

The scheme of the complete Wassermann test is shown in Table LII, including all the controls which would ever be needed. Actually of course, the necessary controls are carried out with the first batch of tests each day, and subsequent tests that day contain usually only three tubes for each unknown blood.

TABLE LII. SCHEME FOR THE COMPLETE WASSERMANN TEST

Tube No.	Serum (or control)	Amount of serum, cc.	Saline	Complement (1 unit in 0.35 cc.), cc.	Antigen (opt. dil.), cc.		Sensitized cells, cc.	Unsensitized cells (10%, cc.)		Expected results (hemolysis)
1	Unknown	0.04	Enough to give a final volume of 1 cc. (± 0.05)	0.7	0.1	Water bath at 37°C. for 60 minutes	0.2		Water bath at 37°C. for 30 minutes	+ or − or ±
2	Unknown	0.02		0.7	0.1		0.2			+ or − or ±
3	Unknown (anticomp. control)	0.06		0.7	—		0.2			+
4	Unknown (hemolytic control)	0.04		0.7	—		—	0.1		−
5	Known positive	0.04		0.7	0.1		0.2			−
6	Known positive	0.03		0.7	0.1		0.2			−
7	Known positive	0.02		0.7	0.1		0.2			−
8	Known positive	0.015		0.7	0.1		0.2			−
9	Known positive	0.01		0.7	0.1		0.2			±
10	Known positive (anti-comp. control)	0.06		0.7	—		0.2			+
11	Known negative	0.04		0.7	0.1		0.2			+
12	Known negative	0.02		0.7	0.1		0.2			+
13	Known negative (anti-comp. control)	0.06		0.7	—		0.2			+
14	Antigen control	—		0.7	0.2		0.2			+
15	Hemolytic system control[a]	—		0.7	—		0.2			+
16	Hemolytic system control[b]	—		0.53	—		0.2			+ or ±
17	Hemolytic system control[c]	—		0.35	—		0.2			±
18	Lysin control	—		—	—		0.2			−
19	Cell control	—		—	—		—	0.1		−

The first three tubes constitute the usual Wassermann test. The "quantitative Wassermann" includes also five additional tubes containing successively smaller amounts of the unknown serum. The six-tube control with known positive serum and the system control must be included once in each day's batch of tests (not in each test, of course). The other controls may be included if desired. Tubes 4 and 18 are usually omitted.

The reagents are added in the order given; it is considered that fixation is stronger if complement is present when antigen and antibody first come together (see page 294).

[a] With 2 units of complement. [b] With 1.5 units of complement. [c] With 1 unit of complement.

It will be noted in the tables that the complement is used in various dilutions for different titrations, so that the volume containing a unit varies somewhat. This is done solely for convenience in pipetting, and the same dilution can be used throughout, except that correspondingly more saline will have to be added in the later tests. (In Table LII, some of the tubes, if filled as directed, may contain as much as 0.04 cc. more than 1.00 cc., but a variation of this magnitude is of no practical importance.)

Interpretation of Results

Failure to obtain hemolysis in the first two tubes containing the unknown serum, if all the controls are satisfactory, indicates the fixation of complement by the reaction between the serum and antigen. For some experimental work with complement fixation, it is desirable to record the degree of fixation quantitatively, and this may be done by comparing the degree of hemolysis with standards, prepared from mixtures containing the same reagents (except that the complement is first heated to inactivate it, and a certain portion of the cells are lysed with water before being added). See, for example, (2). Some also use an arbitrary scale for reporting the Wassermann reaction, or a scheme such as that of Citron, where the reactions are reported as ranging from 4+ to 0, depending upon the degree of hemolysis in tubes 1 and 2. The American Committee on the Evaluation of Sero-diagnostic Tests for Syphilis, however, recommends reporting reactions only as positive, doubtful, or negative.

9. Flocculation Tests for Syphilis

A large number of flocculation tests, all based on the original method of Sachs and Georgi, have been developed. The principle is discussed above on page 260. Complete descriptions of the procedures and preparation of reagents will be found in, for example Kahn (66) and Eagle (29). The Kahn test is one of the best known, and will be described here as typical.

10. The Kahn Test

The antigen is prepared for this reaction from dry powdered beef hearts. This is available commercially, and so is the extract. To make the extract, the powder (25 g.) is extracted four times at ten-minute intervals with 75-cc. portions of ether, then dried. It is then extracted for three days with five cubic centimeters of 95% alcohol for each gram of powder. The mixture is shaken and filtered and, to sensitize the reagent, six milligrams cholesterol is added to each cubic centimeter of filtrate.

The antigen must be titrated to determine the smallest amount of saline which, added to one cubic centimeter, produces aggregates capable of apparently complete dispersion upon the addition of further saline. The amount of saline may be found to be somewhere around one cubic centimeter and usually remains constant for any particular lot of antigen. This amount is added each time it is desired to make ready a batch of antigen for an actual test. The reagents are put into clean, short wide-mouthed vials or tubes, the saline poured into the antigen, and the mixture poured back and forth quickly six or more times without draining the tubes.

The diluted antigen is allowed to stand ten minutes, thoroughly shaken, and three different amounts (0.05, 0.025, and 0.0125 cc.) are pipetted into 75 × 10 mm. test tubes, using small pipets, which should be brought each time into contact with the bottom of the tube, so no reagent will adhere to the walls and not be mixed with the serum. Then 0.15 cc. of inactivated patient's serum is added to each tube. Known positive and negative sera, and an antigen control prepared by adding 0.15 cc. of saline instead of serum, must be included in each series of tests. The racks of tubes are shaken vigorously for three minutes, then one cubic centimeter of saline is added to the first tube and 0.5 cc. to the other two. After thorough shaking of the tubes the results can be read immediately. Final reports are based on readings made after the tubes have stood 15 minutes at room temperature. The following series of reactions may be distinguished:

(4+) Definitely visible particles in clear or opalescent medium. Individual particles readily visible without lifting tubes from the rack.

(3+) Particles definitely visible, not always without examining the tubes individually; less clean-cut.

(2+) Finer particles, frequently in a somewhat turbid medium. They cannot be distinguished without individual examination of the tubes.

(1+) Still finer particles in somewhat turbid medium, seen when slanted tube is shaken.

(Doubtful) Particles just within visible range in somewhat turbid medium.

(0) Medium is transparent, opalescent, and free from visible particles. No turbidity.

11. COLD AGGLUTININS

We may just mention here that tests for "cold agglutinins" (85) in atypical pneumonia, and heterophil agglutinins in infectious mononucleosis (71, 98) have definite diagnostic value in many cases. Hirst (51–53) has shown that the influenza virus agglutinates the red cells of certain species, and that this action is inhibited by antivirus antibody, thus providing an *in vitro* method of titrating such antibodies (see 22, 88). It has even been suggested that the agglutination observed in infectious mononucleosis is due to the virus itself.

12. PHAGOCYTOSIS AND OPSONIC INDEX

A recent modification of Wright's method, due to Lyons (80), is used especially in selecting donors for immunotransfusion.

E. *In Vitro* Experiments (Theoretical)

1. OPTIMAL PROPORTIONS DETERMINATION

The significance of this determination has been discussed in Chapter II. We may repeat that it is a convenient way to estimate antibody concentrations roughly.

Unless something is known about the strength of the serum it is usually necessary first to locate the optimal proportions point approximately by a rough test. The original technic of Dean and Webb (24) has been modified by a number of workers; the technic described in (14) will be given here. The technic of making serial dilutions is described on page 414.

Prepare in Wassermann tubes serial dilutions (successively 1:2 or 1:3) of the antigen, starting with a solution of known concentration. Either make these dilutions so as to leave 0.5 cc. in each tube, or transfer 0.5 cc. of each to a series of tubes as the dilutions are being made. Dilute enough serum for the rough test 1:5 or 1:10, depending on its probable strength. Have ready a little rack of small tubes of 1-cc. capacity (see page 397) in a water bath at 37°C. (or if greater speed is desired, at 40° or 45°), each tube being one-third immersed in the water. With a 1-cc. pipet add 0.5 cc. of the diluted serum to the highest dilution of the antigen, immediately mix by brief shaking, then immediately remove the mixture from the original tube and transfer to one of the 1-cc. tubes, using a capillary pipet with a long, not too fine tip. This insures thorough mixing. Replace the small tube in the bath. Record the time, to the nearest quarter-minute. Proceed similarly with the other antigen dilutions, using the same capillary pipet each time, and going from the higher dilutions to the lower (from weak to strong antigen).

Observe the tubes continuously. Cloudiness will begin to appear in the tubes which are going to flocculate, usually within a few minutes. Watch for the point at which individual particles just become visible to the unaided eye (this point varies with different observers, but is more or less consistent for any one individual). Record the time of particulation in each tube, until four or more tubes have flocculated, or it is clear they are not going to.

TABLE LIII

DETERMINATION OF OPTIMAL PROPORTIONS POINT

	Volumes (in cubic centimeters)								
Diluted antigen	0.5	0.4	0.35	0.30	0.25	0.20	0.17	0.15	0.12
Saline	—	0.1	0.15	0.20	0.25	0.30	0.33	0.35	0.38
Diluted serum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of particulation (min.)	15	6.5	3	2.5	3	5	13.5	—	—

The diluted antigen and saline are mixed first, then the diluted serum added to one tube at a time, beginning at the right; the time each mixture is placed in the water bath is recorded. The time of particulation is similarly recorded for each tube.

The optimal proportions point may be expected to lie somewhere between the values indicated by the two most rapid tubes. From the antigen and antibody dilutions present in these tubes, it may be calculated what would be a suitable dilution of antigen and antibody for the "fine test." The serum dilution should be such that flocculation at the optimum may be expected to take place in five to ten minutes. Make sufficient diluted antigen and diluted serum for the following procedure.

In each of a series of Wassermann tubes place the amounts of the antigen dilution shown in Table LIII, and add the indicated volumes of saline to bring the total in each case to 0.5 cc. Then add to each tube in turn 0.5 cc. of the diluted serum, beginning with the weakest antigen, and mixing, transferring, and timing as before. If the dilutions used were the correct ones, one tube, not at either end of the series, will show most rapid flocculation, or occasionally two or more tubes will run a "dead heat." If the fourth tube from the left in Table LIII, for instance, was most rapid, when we used a 1:400 dilution of stock antigen and a 1:10 dilution of antiserum, we should calculate that the optimal ratio of antigen to serum dilution was $0.5/0.3 \times 400/10 = 66.7$.

From a knowledge of the actual concentration of the stock antigen, it may be calculated how many milligrams of antigen, or antigen nitrogen, react optimally with 1 cc. of serum. For example, if in the above case, where we found an optimal ratio of 66.7, we had started with a stock solution containing 1.25 mg. of protein nitrogen per cubic centimeter, we should calculate that 1 cc. of antiserum reacts optimally with $1/66.7 \times 1.25 = 0.019$ mg. of protein nitrogen. Or conversely, that to react with one milligram of antigen nitrogen, $66.7/1.25 = 53.4$ cc. of serum would be needed.

2. Ramon Titration

This is essentially the same titration, and is historically earlier, but need not be described in so much detail, since the Dean and Webb titration just described is more generally useful when rabbit sera are being studied. In the Ramon titration, constant dilutions of antigen are tested with varying dilutions of serum (see above, Chapter II, page 70).

The amount of toxin which gives most rapid flocculation with one standard unit of antitoxin is first determined. This amount is designated as the L_f unit. Then unknown antisera can be titrated against this standardized toxin, remembering that the toxin will gradually change in strength and consequently will need restandardization at intervals. Ordinary toxin is stable for flocculation tests for at least several years, if kept cold. It may be less stable as judged by *in vivo* tests.

A sample titration is shown in Table LIV. Descriptions of the technic of the Ramon titration may be found in papers by Ramon (102), and Glenny and Okell (37).

3. Determination of Equivalence Zone

This has been defined above as the zone in which antibody and antigen are in equivalent proportions, so that when they are mixed, allowed to react completely, and the supernatants are examined, neither antibody nor antigen, or perhaps traces of both, will be found in tubes containing such mixtures. The equivalence zone varies considerably in width; the point of optimal proportions in some systems usually falls within this zone; in other systems it may fail to do so. In such systems it may be necessary to locate the zone approximately by rough preliminary tests.

Assuming that we are dealing with a system (antigen and homologous serum) where it is likely that the equivalence zone will include the optimum, mix diluted antigen, saline, and serum in the ratios shown in Table LIV, but use undiluted serum, and a dilution of the antigen to correspond. That is, if in Table LIV the serum dilution was 1:10, and the antigen dilution 1:400, in the present experiment we should use straight serum and antigen diluted 1:40. Mix the reagents by shaking, but leave them in the same tubes. Place in a water bath until precipitation is complete, then in the ice box overnight.

TABLE LIV

RAMON TITRATION OF ANTITOXIN

	Amounts of reagents (cubic centimeters)								
Serum, dil. 1:5	0.07	0.08	0.09	0.10	0.11	0.12	0.13	0.14	0.15
Toxin	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Time of flocculation, min.	—	—	120	40	19	15	20	45	—

If the toxin contained 15 L_f units per cubic centimeter, we may calculate that 1 cc. of the above serum would neutralize $1/0.12 \times 5 \times 15 = 624$ L_f units of toxin.

Then centrifuge, take off the supernatants, and test for antibody and antigen. The test for antigen is made by the interfacial technic (see page 421), placing straight serum in the bottom of the little tubes, and overlayering with the supernatants. The test for antibody is made in the same way, placing the supernatants in the bottoms of the tubes, and overlayering with a dilution of antigen about the same as that in the last mixture in the table. The results shown in Table LV might be obtained.

In the instance shown in the table, we should say the equivalence zone extended from $0.50/0.35 \times 40 = 57.2$ to $0.50/0.20 \times 40 = 100$, in terms of ratios of antigen dilution to serum dilutions (compare with optimal ratio, page 436 and Table LIII). If we wished we might define the equivalence point as the midpoint of the equivalence zone, and take it to be 80, but it is clear that this is a rather arbitrary procedure, especially as the midpoint would differ in different experiments, depending upon the steps of dilution used. Also, it must be remembered that the test for antigen in the supernatants is much more delicate than the test for antibody. The difference can be lessened somewhat by using in the latter connection the gelatin technic of Hanks (41).

TABLE LV

DETERMINATION OF EQUIVALENCE ZONE

Component	Volumes (cubic centimeters)								
Diluted antigen (1:40)	0.5	0.4	0.35	0.30	0.25	0.20	0.17	0.14	0.12
Saline	—	0.1	0.15	0.20	0.25	0.30	0.33	0.36	0.38
Serum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mix, incubate, keep overnight in the ice box, test supernatants									
Reaction with antiserum	+	+	+	0	0	0	0	0	0
Reaction with antigen	0	0	0	0	0	0	±	+	+

4. PREPARATION OF SYNTHETIC ANTIGENS

The following example of the coupling of diazotized arsanilic acid with the proteins of horse serum may be taken as typical of methods of preparing chemically modified antigens.

Weigh out one gram of arsanilic acid for each gram of protein *nitrogen* present in the amount of serum it is desired to use (the amount for pure proteins, if the tyrosine and histidine content is known, can be calculated more precisely by the formula of Boyd and Hooker (16). One gram dissolves in about 70 cc. of water, with the addition of 1.2 cc. of concentrated hydrochloric acid. Cool in a freezing bath (ice and salt), diazotize by the addition of the requisite amount (about 9 cc.) of cold 0.5 *N* sodium nitrite solution, using starch–iodide paper as an indicator. Allow to stand about 30 minutes before the final test with the paper. Add one gram of urea, mix, and allow to stand ten minutes. Pour the mixture into the cold serum, add enough cold alkali (sodium hydroxide or trisodium phosphate) to make the mixture slightly alkaline to litmus, allow to stand in the ice box overnight. Coupling, as shown by the development of a red color, should begin within a few minutes, and should be complete next morning.

Proteins coupled with arsanilic acid can usually be precipitated easily by the cautious addition of hydrochloric acid, and this fact may be utilized for purification. The proper amount may be determined by trials with 1 cc. samples of the mixture, or the concentrated acid may be added drop by drop with shaking until the maximum of precipitate is formed. Other modified proteins, not precipitable in this way, may be purified by adding sodium or ammonium sulfate, preferably the former because it contains no nitrogen, in conjunction with enough acid to bring the protein near its (often rather acid) isoelectric point. Filter off the precipitate on paper, or centrifuge it down and remove liquid. Mix thoroughly with water, then cautiously add alkali to bring the material into solution, being careful to crush all lumps with a stirring rod. Filter. Repeat the precipitation, solution, and filtration two or three times. If the protein is to be injected, the final solution should have salt added to make it isotonic and should be sterilized by passage through a Berkefeld or Seitz filter.

Purification of such conjugated antigens can also be effected by repeated precipitation with alcohol in the cold, followed by a final dialysis to remove residual alcohol.

5. Specific Inhibition

The principle of the inhibition of serological reactions by the appropriate haptens has been discussed on page 114. If the inhibitive power of a number of related haptens is to be compared, it is best to do the inhibition in a quantitative manner, using definite amounts of hapten. It is convenient to make solutions of the various haptens 0.02 *M* or 0.01 *M*, and use successive dilutions of these. The dilutions may increase successively by a factor of 2, or in very fine work, 1.5.

A fixed amount of each of the hapten dilutions is mixed with a constant volume of serum. After a preliminary incubation period (which may be unnecessary), these are now tested by the usual precipitin technic, against an appropriate dilution of the appropriate antigen. To make the test more delicate, the antiserum may be diluted 1:4 or 1:8, and the gelatin technic of Hanks (41) used. This is illustrated in the following experiment (59) on the inhibitive power of various derivatives of strychnine against an antiserum prepared by injecting hemocyanin coupled with diazotized aminostrychnine.

A roughly optimal concentration of monoaminostrychnineazocasein, about 20 micrograms of nitrogen per cubic centimeter, was prepared in 5% gelatin in saline solution. A column of this mixture, about 0.2 cc., allowed to harden in tubes of 6-mm. bore, was overlayered with 0.2 cc. of a solution containing 0.025 cc. of pooled antisera and an amount (1.0–0.017 micromoles) of one of the substances tested for inhibitive capacity. The tubes were then kept in the incubator at 37°C. for five hours; reactions were recorded at hourly intervals, and after overnight refrigeration. With the lengthening of time of incubation, increasingly larger amounts of inhibitors are required to prevent the appearance of a visible precipitate at the interface. Uninhibited serum gave a prompt clouding at the interface. Typical results are shown in Table LVI.

TABLE LVI

TYPICAL RESULTS OBTAINED IN AN INHIBITION EXPERIMENT (59). PRECIPITATION OF CASEIN–STRYCHNINE COMPOUND BY ANTI-STRYCHNINE SERUM MIXED WITH VARIOUS SUBSTANCES

Test substance (dissolved as the hydrochloride)	Micromoles of test substance										
	1.00	0.67	0.44	0.30	0.20	0.13	0.088	0.058	0.039	0.026	0.017
Strychnine[a]			–	–	–	–	–	–	t	±	+
Mononitrostrychnine[a]			–	–	–	–	–	–	t	+	+
Dinitrostrychnine[a]			C	C	C	C	C	+	+	+	+
Monoaminostrychnine	–	–	–	–	–	–	t	t	+	+	
Diaminostrychnine	–	–	–	–	–	–	–	t	t	t	
Brucine[a]	C	C	–	–	–	–	t	±	+	+	
Morphine	+	+	+	+	+	+	+	+	+	+	+
Quinine	+	+	+	+	+	+	+	+	+	+	+
Tryptophan	+	+	+	+	+	+	+	+	+	+	+
Nicotinic acid	+	+	+	+	+	+	+	+	+	+	+

C indicates a cloudiness of the supernatant, which developed in mixtures of the particular test substance with antiserum or with normal serum. + indicates precipitation at interface, – absence of precipitation, t trace.

[a] indicates that the substances so designated were actually used in amounts 1.14 times greater than stated. Because of the limited quantitative precision of the test, this difference is not significant.

6. ANALYSIS OF SPECIFIC PRECIPITATES

If the precipitates are made in the part of the range where all the antigen is precipitated, the composition of the precipitate can be determined by analyzing it for nitrogen. The value obtained, minus the nitrogen contained in the antigen (if any), gives the antibody nitrogen, and the quotient gives the ratio of antibody to antigen (in terms of nitrogen) in the precipitate. In the case of nitrogen-free carbohydrates no correction for antigen nitrogen is required. If the antigen is not all precipitated, it is necessary either to have some method of measuring the residual antigen in the supernatant (see 48, 49, 68), or to use an antigen containing some characteristic making it possible to measure it independently of nitrogen determinations. This method was first applied by Wu and his collaborators (130, 131) to the cases where the antigen consisted of hemoglobin and iodoalbumin, respectively. Heidelberger and Kendall (47, 48) made use of colorimetric estimation of a dye–antigen; Kurotchkin and Kratze (70) estimated a carbohydrate antigen by determination as reducing sugar. Artificial antigens containing arsenic have been used (45, 56, 84).

Of the natural protein antigens, the hemocyanins are particularly suitable for such studies, since they are very good antigens, unlike hemoglobin, and contain copper which can be used for their estimation in precipitates (58, 83). This technic may be given as an example.

The nitrogen in the precipitates is determined by the micro-Kjeldahl method of Parnas and Wagner (97). In various laboratories a number of modifications of this method, some of which do not appear to have been published, are in use. Of these perhaps the most helpful is the use of saturated boric acid solution to absorb the ammonia as it distills over, followed by direct titration (with methyl red) of the ammonia with $N/70$ (or 0.01 N) hydrochloric acid using a blank as color standard. This is more convenient and avoids errors due to carbon dioxide from the air. The copper is determined either by the method of Locke, Main, and Rosbash (75) or McFarlane (81). In the author's laboratory a modified method combining certain features of both is in use. Sodium hydroxide instead of ammonia is used, and by making the readings on the Evelyn (micro) photoelectric colorimeter, the amount of amyl alcohol can be cut to 3 cc. with resulting increase in sensitivity. More delicate methods of determining copper have since been developed.

Using these methods, it is convenient to make the precipitates for nitrogen analysis so that they will contain a total of about 1 mg. of nitrogen. If the ratio of antibody to antigen in the equivalence zone is not already known, it may be estimated roughly for the first experiments from the theoretical relation with molecular weight of the antigen proposed by Boyd and Hooker (16, 17). From two to three times as large a precipitate will be needed for the copper determinations, which should be done on a precipitate containing about 0.015 mg. of copper. Such precipitates are prepared by mixing antibody and antigen in the same proportion as for the corresponding nitrogen precipitates, but in amounts correspondingly greater. In the region of antigen excess, the copper precipitates will have to be made with larger and larger amounts of antigen, as only part of the antigen will be precipitated. It is desirable, if sufficient serum is available, to do all the analyses in duplicate or triplicate.

Any proportion of reagents may be selected arbitrarily as a reference point, and other mixtures expressed in terms of this, or the optimal proportions point or the equivalence point (page 438) may be determined and used for this purpose. Then mixtures containing proportionately less or more antibody might be expressed as being made with, say, 70%, or 150%, of the optimal antibody (83).

The analytical results may be reduced to some convenient standard, such as the yield from 1 cc. of serum, or from 1 mg. of antigen nitrogen, and from a knowledge of the nitrogen/copper ratio in the hemocyanin, the copper figures translated into equivalent antigen nitrogen. By subtracting this from the total, the antibody nitrogen is obtained, and the ratio gives the ratio of antibody nitrogen to antigen nitrogen in the precipitate. Since the per cent of nitrogen in the antibody and in antigen usually do not differ much, the ratio by nitrogen is very nearly the ratio by weight of antibody to antigen. Different ratios are to be expected for each mixture of antiserum and antigen in which the proportions of the reagents differ (see Fig. 31a on page 235).

Heidelberger and MacPherson (50) have described a micromethod by which as little as 10 γ of specific precipitable nitrogen may be determined. They make use of the color produced by reaction of the Folin phenol reagent directly with the dissolved specific precipitate.

7. Specific Absorption

It is sometimes desired to remove one or more antibodies from an antiserum containing a mixture, either to render the serum more specific, or to obtain information about the specificity of the antigen. As an example we may describe the absorption of an anti-human precipitating serum with blood of some other mammal it is found to react with, so as to enable a distinction to be made between the two species in a forensic blood examination (see page 423).

The antiserum is best treated with an amount of the heterologous antigen which is optimal according to the Dean and Webb titration (page 436). The amount added to the antiserum should not exceed one-half the original volume of the serum. The mixture of antiserum and heterologous antigen is allowed to stand in the icebox all night, and the precipitate is centrifuged off. Generally it will be found that the fluid remaining will no longer react with the heterologous antigen (or with antigens closely related to it), while retaining a practically undiminished power of reacting with the homologous antigen. If a heterologous reaction is still found treatment with about one-fourth the first amount of heterologous antigen will usually remove it. The titer of the absorbed antiserum must then be determined afresh against the homologous antigen.

Absorption of undesired agglutinins is necessary in the preparation of specific immune agglutinating sera for human blood group factors M and N (see page 414). Details will be found in (111).

Specific absorption is also useful in studies on bacterial agglutination. For instance, in the identification of bacteria of the enteric group, it will often be found that the agglutinating serum prepared by infection or artificial inoculation of an animal agglutinates not only the strain used in its preparation, but also related organisms. Agglutinins for one or more of these organisms can be removed by absorption (see Table XV). The serum is treated with a heavy suspension of the appropriate organisms, incubated two hours at 37°C., and the bacteria centrifuged off. If sufficient suspension were used, and other conditions were right, agglutinins for that organism will be found to have been removed, while agglutinins for the injected organism and possibly other related organisms, will remain. If absorption is not complete it can be repeated.

F. Animal Experiments

1. INJECTION AND BLEEDING

These technics have already been described on page 400.

2. ANAPHYLAXIS

Guinea pigs may be sensitized actively by a single injection (by almost any route) of an antigen. As little as about 7×10^{-8} g. of protein has been found to sensitize somewhat, but it is better, especially if it is desired to demonstrate fatal shock, to use larger amounts, such as 0.001 g. Passive sensitization may be produced by intravenous injection of antiserum; as little as 0.1 cc. of a (rabbit) precipitating serum of high titer may be sufficient. Active sensitization is maximal in about three weeks, passive sensitization after a few hours. These matters are discussed above, page 312.

Shock is produced by intravenous or intracardiac injection of the antigen, using considerably more than was used for sensitization, for example, about 0.4 cc. of horse serum or undiluted egg albumin solution. The symptoms of anaphylaxis are described on page 314.

3. Titration of Antisera, Toxins, etc.

In many cases the only practical way of estimating the potency of a toxic or infectious preparation, or the protective power of an antiserum, is by injection of graded doses into test animals (see 74). Our first problem is: how best to estimate the strength of a preparation (e.g., toxin or antitoxin) by animal experiments? We came across such questions at the beginning of this book, and in the discussion of the determination of the toxicity of toxins, etc. It is now time to see how we may obtain an answer. To do so will involve certain elementary mathematical considerations, which, although frequently neglected by immunologists, are almost essential to the proper planning or interpretation of biological experiments.

Basic Statistical Principles

If we were testing a protective serum, and found that one dose of it would protect every animal against several average lethal doses of a toxic or infectious agent, even when the tests included hundreds of animals, it is clear that we should be justified in concluding without further ado that the serum had a high protective power. But considerations of time and expense usually prevent us from using so many animals in an experiment, and the results in any case are seldom so dramatic and satisfying as the hypothetical ones just mentioned. More probably, we shall find experiments cropping up in which not all the treated animals are protected, and not all the controls die. Even if our problem is of the simplest possible sort, i.e., simply to decide if one preparation is more potent than another, we have to decide when to conclude that the results indicate a significant difference in potency, and what degree of confidence we are justified in feeling in them. In planning an experiment, we must decide on the basis of the expected behavior of the reagents and our knowledge of statistical principles, how many animals will be required to give a satisfactory answer to the question we are asking.

The procedure is always to compare the actual results with those to be expected on the basis of pure chance. That is, we assume provisionally that the treatment has made no difference in the life expectation of the treated animals, and therefore the most likely result of the experiment would be that equal numbers of both the treated and the control groups would die. But it is also possible for more of the controls than of the treated group to die, also by pure chance. If nothing but chance is operating, we can calculate mathematically how frequently this would happen.

Suppose that we know accurately, from experiments with large numbers of animals, that a certain dose of a toxin (or whatever agent we are using) will kill just 30% of our animals. We express this by saying that the probability of death = 0.3. Since the animal must either live or die, the probability of living = 1.00 − 0.3 = 0.7. We may symbolize the chance of death by p, and the chance of living by q; we shall always have: $p + q = 1$.

Suppose our experiment includes only two animals, which we may refer to as A and B. The chance that A will die is p. In the absence of disturbing influences, the chance that B will also die is $p \times p = p^2$. Similarly we may calculate the chances of the other possible outcomes:

A lives and B lives:	q^2
A lives, B dies:	pq
A dies, B lives:	pq
A and B both die:	p^2

Therefore we obtain:

chance of 2 deaths:	p^2
chance of 1 death:	$2pq$
chance of no death:	q^2

These values are the successive terms of the binomial expansion of $(p + q)^2 = p^2 + 2\,pq + q^2$, and it is easy to show that when more than two animals are involved, the chances of the various outcomes are calculated similarly, from the expression $(p + q)^n$, where n = the number of animals. We can now compare these frequencies, predicted on the basis of pure chance, with the observed results.

If n is at all large the computations become laborious, but fortunately, since we are usually not interested in the actual probability of each possible result, but only wish to know if a given observed result is likely or unlikely, we can make use of a short cut. If n is large, the curve obtained by plotting, against the probability of each possible outcome (0, 1, 2, 3, etc. deaths), the *number* of deaths, is the normal curve of error, or probability curve, which has a known formula. We know that this curve has a certain simple function, called the standard deviation, and represented by σ, which has this property: if we lay off distances equal to σ on each side of the center (mean) of the probability curve, and draw vertical lines, these lines include between them about two-thirds of the area under the curve; or in other words, the probability of a result represented by a point outside these lines is only about one-third (see Fig. 48).

We may calculate also the probability of results corresponding to deviations from the mean corresponding to 2σ, 3σ, or any other multiple we like. Therefore, if we observe a given deviation in an experiment (that is, our observed result is not the one predicted as most probable on the basis of pure chance), we may calculate, or determine from tables, just how probable it is, if it is assumed to be one of the rare, but possible, chance outcomes. This probability is never zero. Just how small it must be before we decide that the result is too *improbable* for the result to be solely due to chance, is a matter of individual taste, and may depend also upon the type of experiment. We might be more readily convinced, for instance, in an experiment on the protective effect of an antiserum, than in an experiment apparently demonstrating the existence of telepathy or clairvoyance. If we do not believe the departure from expectation is due to chance, then we must conclude, in a properly designed ex-

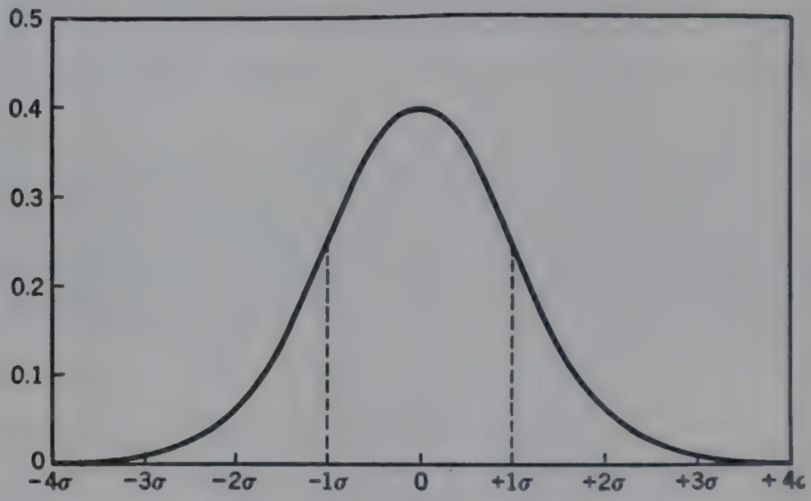

Fig. 48. Probability curve, showing portion of area included by lines drawn through $+\sigma$ and $-\sigma$ (σ being the standard deviation).

periment, that the experimental treatment is responsible for the deviation, and that the preparation we are testing is not without effect. In biological experiments in general it has proved satisfactory to consider as significant an experiment showing a deviation of as much as 2σ, which would be expected on the basis of pure chance only about once in twenty times. This will mean that we shall be led to follow up a false indication once in every 20 experiments, but has the advantage that we shall not miss a lead into a valuable investigation so often as if we set our standard of significance higher and required a larger deviation.

When the probabilities p and q are known, σ may be calculated very simply from the relation, $\sigma = \sqrt{npq}$. Thus in the hypothetical experiment above, if we treated 20 animals, σ would equal $\sqrt{20 \times 0.3 \times 0.7} = 2.05$. Since this gives for 2σ the value of 4.1, this means that in such an experiment we should consider a result significant if the deaths observed differed from the true average number of deaths, six, by more than four; that is, if only one or none of the animals die we should be led to follow up the experiment with a more elaborate one in the hope of proving that the treatment really had protective value. Tables for such tests will be found in references given below.

Estimation of Standard Error

In the majority of experiments we do not know the true expectations, p and q; consequently we cannot calculate σ from them, and must *estimate* it from the data of the experiment itself. This is possible, if the set-up includes controls. The size of σ, and therefore the magnitude of the deviation which we must find to consider it significant, will depend upon the number of animals used. Details of such calculations will be found in (117). We shall not give any examples here, for other methods will prove better adapted to our purposes.

It should be pointed out that σ as we thus calculate it is merely the standard error of response, and if the response is to be used to predict the proper dose of an antitoxin, for instance, other factors enter into the estimate of the error. One of the most important of these is the slope of the dosage–mortality curve (in its straight line form). Formerly ignored, such facts are taken account of in more modern methods of treating the problem, such as those mentioned below.

If the number of animals is small, as it usually is, there is a better way than the above of measuring the significance of any difference in mortality between treated and control animals. This is called the χ^2 test, and is based on a rather simple calculation from the data, which can be made without any understanding of the mathematical theory, which is not presented here.

The χ^2 Test

The χ^2 test was specifically designed to test whether an observed distribution of individuals in different categories can be due solely to chance. For example we may study the figures on the incidence of typhoid among inoculated and uninoculated troops, collected by the British Antityphoid Committee and analyzed by Greenwood and Yule (40). See Table LVII.

TABLE LVII

TYPHOID INCIDENCE IN INOCULATED AND UNINOCULATED TROOPS (40)

	Not attacked	Attacked	Totals
Inoculated	10,322 (10,202)	56 (176)	10,378
Not inoculated	8,664 (8,784)	272 (152)	8,936
Totals	18,986	328	19,314

Numbers in parentheses are those expected in each category if the inoculations had no effect (see text).

To compute χ^2 from these data, we first calculate the numbers to be expected in each category, on the assumption that the inoculations did not influence the susceptibility to typhoid of those who received them. This is done from the marginal totals. A total of 19,314 persons was studied, and the total number attacked was 328, or 328/19,314 of all those studied. If the inoculations had no effect, we should calculate that on the basis of pure chance we should find 328/19,314 times the total inoculated, or $328/19{,}314 \times 10{,}378 = 176$ to be attacked, as well as the proportional number, $328/19{,}314 \times 8{,}936 = 152$, of the uninoculated. The other expectations are calculated similarly. Then each expected value is subtracted from the observed, or vice versa, depending on which is larger. These values are the deviations, which we may represent by d. We now compute χ^2 by squaring each deviation, dividing by the expected value for that category, and adding the four results so obtained. This may be symbolized mathematically by

$$\chi^2 = \Sigma(d^2/m)$$

where *m* is the value expected in each case. The calculations are shown in detail in Table LVIII.

For the results of Table LVIII we obtain, $\chi^2 = 179.61$. We can find the probability of such a value by looking in tables such as those given by Pearson (99), Fisher (32), Fisher and Yates (33), taking account of the number of "degrees of freedom," which in 2 × 2 tables such as Table LVII is always one. For other experiments the degrees of freedom may be calculated by ascertaining how many of the "cells" of the table could be filled in arbitrarily and still leave enough empty so that, by filling the remainder in properly, the original marginal totals would remain the same. (There are four cells in a 2 × 2 table, six in a 3 × 2 table, etc.) It is clear that in a 2 × 2 table like the present one, only one cell can thus be filled in arbitrarily. From such tables we find that the probability of the result shown in Table LVIII is less than 0.0001, so that the chances that the observed difference in favor of the inoculated soldiers (Table LVII) is purely accidental are less than one in 10,000. We may regard it as certain that the inoculations played a definite role in cutting down the incidence of typhoid.

TABLE LVIII

CALCULATION OF χ^2

$$\begin{array}{rcr}
10{,}322 - 10{,}202 & = & 120 \\
8{,}664 - 8{,}784 & = & -120 \\
56 - 176 & = & -120 \\
272 - 152 & = & 120 \\
\dfrac{(120)^2}{10{,}202} & = & 1.41 \\
\dfrac{(-120)^2}{8{,}784} & = & 1.64 \\
\dfrac{(-120)^2}{176} & = & 81.82 \\
\dfrac{(120)^2}{152} & = & 94.74 \\
\text{Total } (= \chi^2) & = & 179.61
\end{array}$$

If the numbers involved are small, so that the number expected in any class is less than ten, a "correction for continuity" should be applied to the simple calculations just outlined, for greatest accuracy. This is particularly desirable if the results appear to be just barely significant (probability slightly less than 0.05). A more accurate method of making this correction is given by Fisher and Yates (33).

Application of the χ^2 test to immunological experiments, even to some of the results which have been published as showing the advantage of some certain mode of treatment, sometimes gives surprising results. For instance, if out of 73 animals given a serum only 28 died, while out of 13 controls 9 died, it is probable that some workers would consider that a definite effect of the serum was indicated. Actually, however, accurate application of the χ^2 test shows that the results are below our usual level of significance (33).

"Randomizing"

It will be noted that in all the examples discussed above, we are assuming that there are no differences between our treated and control groups except those produced by the treatment. In reality, however, animals are of course never exactly alike, nor exposed to exactly the same environment, particularly before being put under test conditions. It is obvious that if we should give our treatment to a group, all or most of which happened to differ in some respect from the controls, we might find an apparently significant difference in results which would be due, not to the treatment, but to lack of uniformity in the animals. It is essential in good experiments to avoid such disturbing factors as much as possible. This involves the use of dice, or some chance element, to assign the different animals and groups to the different treatments, and the use of arrangements such as randomized blocks and "Latin squares," so that duplication can be avoided (31, 33).

For example, if a number of animals were to be tested in succession for susceptibility to nonlethal doses of a number of different toxins (or infectious agents), the different animals should be given the different toxins in different orders, so as to make it possible at the end of the experiment to show that any greater or lesser susceptibility to treatments given near the end of the series was not due to the earlier treatments. Methods of doing this properly will be found in a book by Fisher (31). The results of actual immunological experiments are discussed from this point of view by Topley and Wilson (117) and Irwin and Cheeseman (64).

Fifty Per Cent End Point

It has been shown that the greatest accuracy, with most economy in the use of animals, is obtained if we try to find for a toxin, not the dose which will kill all animals, but the dose which will kill 50% of them (34). This is sometimes called the LD50. If we were testing the protective power of a serum, we should try to determine the dose which would just save 50% of animals, each of which had received a fatal dose of the agent against which the serum was supposed to protect. There are a number of ways of estimating the LD50. One of the simplest is that suggested by Reed and Muench (103).

TABLE LIX

TITRATION OF A TOXIN (METHOD OF REED AND MUENCH)

(*a*) Dose (mg.)	(*b*) Lived	(*c*) Died	Σ		(*f*) Per cent mortality
			(*d*) Lived	(*e*) Died	
0.0625	4	1	12	1	8
0.125	3	2	8	3	27
0.25	4	1	5	4	44
0.5	0	5	1	9	90
1.0	1	4	1	13	93
2.0	0	5	0	18	100
4.0	0	5	0	23	100

Column *d* is formed by adding column *b* from the bottom, and entering each subtotal. Column *e* is formed similarly by adding column *c* from the top. The percentage mortality is calculated from columns *d* and *e*.

Method of Reed and Muench

Unless the approximate titer is already known, it will usually be necessary to use a large number of small groups of test animals at different dilutions. Let us suppose that we are testing a toxic fraction obtained from a microorganism, and each group of five mice receives a different amount. Results of a hypothetical experiment calculated by the method of Reed and Muench are shown in Table LIX. When the number of animals in each group is small, as in this example, there will be a number of accidental departures from a regular progression of mortalities. To compensate for this in so far as possible, Reed and Muench proposed computing cumulative mortalities for all the groups in succession. Since it may be assumed that a mouse surviving a given amount of toxin could have survived any smaller amount, the survivors are successively added, beginning at the bottom of the table, for each different dose (column *e*). Deaths are similarly added (column *d*) and the percentage mortality computed at each step (column *f*).

In the present case the point of 50% mortality seems to lie between 0.25 and 0.50 mg. If the percentages of accumulated survivals and deaths are each plotted against the dose, it can be seen from the graph that the 50% point (where the lines cross) is nearer 0.25 mg., about one-seventh the distance from the 0.25- to the 0.50-mg. point. The exact point can be calculated from the formula:

$$\frac{50 - (\%\text{ mortality at amount next below})}{(\%\text{ mortality next above}) - (\%\text{ mortality next below})} = \begin{array}{c}\text{proportional}\\ \text{distance}\end{array}$$

In the present case this gives:

$$\frac{50 - 44}{90 - 44} = \frac{6}{46} = 0.13$$

Since the amounts increase on a logarithmic scale, it is necessary to carry out the final calculations as follows:

$$\begin{array}{lll} \log & 0.25 \text{ (lower amount)} & \bar{1}.3979 \\ + & 0.13 \text{ (propor. dist.)} \times \log 2 \text{ (dil. factor)} & \underline{0.0391} \\ = & \text{sum (log of end point)} & \bar{1}.4370 \end{array}$$

which gives for the end point an amount of approximately 0.27 mg.

If the proper graph paper is available, this calculation can be carried out more quickly graphically, and some prefer to do this. Details will be found in (103).

If another toxic preparation were tested and found to have a 50% end point of 0.13, we should conclude that it was approximately twice as strong as the first.

An application of the above method to the titration of anti-influenza serum has been given by Horsfall (61).

An advantage of the method outlined here is that by adding the animals in each column we effect some equalization of the chance variations in mortality which are always present, and are able to get practically the effect of testing two larger groups of animals with two dilutions between which the end point is known to lie. A serious drawback to the Reed-Muench method is that it gives no estimate of the uncertainty (standard error) of our determination of the 50% end point. We *estimated* the end point (LD50), but did not provide any "yard stick" to enable us to judge how close to the true end point we had come by one experiment involving only 35 mice. In other words, we had nothing analogous to σ (see above). Methods are available by which such an estimate can be obtained, and such methods are of course to be preferred.

Method of Irwin and Cheeseman

As an illustration we may give the approximate method devised by Irwin and Cheeseman (64), for estimation of the standard error (σ) of the LD50. Their method seems to provide as much accuracy as is likely to be required for most such work, and will probably prove easier for readers of the present book to follow than would some of the more accurate (and laborious) methods. We apply their method here to the data of Table LIX.

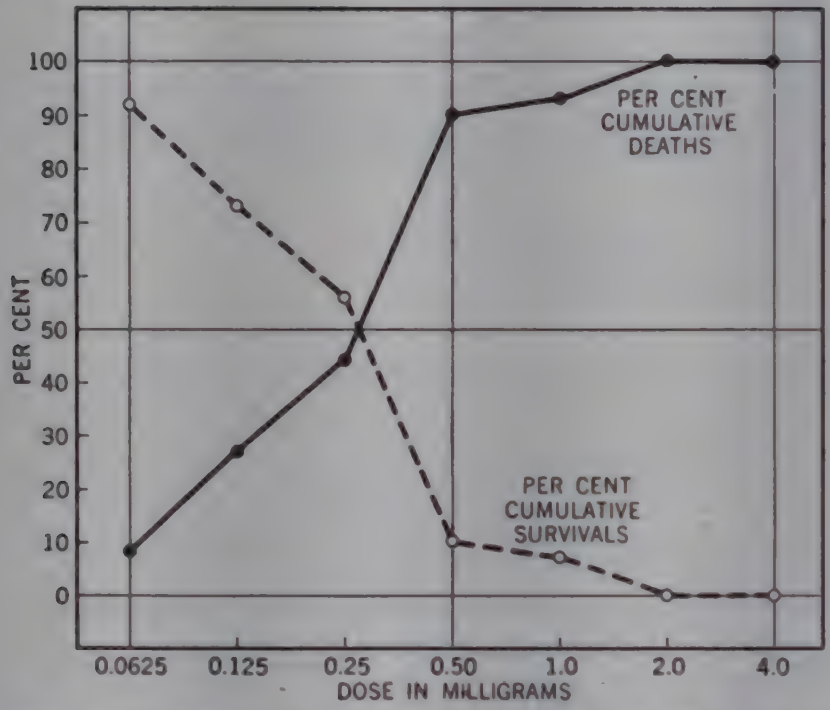

Fig. 49. Graphical illustration of determination of 50% end point (LD50 by method of Reed and Muench. One curve gives percentage of accumulated survivals, the other accumulated deaths (see Table LIX). Interpolated dose (estimated from point of intersection) is the LD50.

First they estimate the LD50, using the method of Kärber (65), which gives 0.233, a different result from that we obtained above by the method of Reed and Muench, a fact which itself emphasizes the uncertainty in all these estimated values.* As before, it is necessary to state the doses in logarithmic form. The actual value of the initial dose enters only into the final computations, so we are at liberty to divide through by this number before taking logarithms.

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* Since the "maximum likelihood" value is 0.219, it is apparent that the Kärber method is superior to the simpler method of Reed and Muench.

Since each dose is twice as large as the preceding, the logarithms will be proportional to the integers, 0, 1, 2, 3, 4, etc., and these are called working doses (Table LX). The procedure is as follows:

(*a*) Write down the deaths and the mortalities for each dose (Table LX). They are given here as decimal fractions, but of course could be written as percentages, as above.

(*b*) Assume the dose previous to the lowest used would have given zero mortality, and take the sum of each pair of mortalities, stopping at the first 100% point beyond any reversals.

Table LX

Calculation of the Fifty Per Cent End Point in the Titration of a Toxin, by the Method of Kärber (see Table LIX)

Dose		Deaths	Mortality	
Actual (mg.)	Working (power of 2)		Actual	Interpolated
				0.1
0.0625	0	1	0.2	
				0.3
0.125	1	2	0.4	
				0.3
0.25	2	1	0.2	
				0.6
0.5	3	5	1.0	
				0.9
1.0	4	4	0.8	
				0.9
2.0	5	5	1.0	
4.0	6	5	1.0	
Total				3.1

Five animals were given each dose.

(*c*) Add these quantities. Result, 3.1.

(*d*) Deduct 3.1 from 5, the last dose included in these calculations. Result, 1.9.

Then the working LD50 = 1.9. The actual LD50 (in mg.) is calculated by the equation:

$$\begin{aligned}\log \text{actual LD50} &= \log (0.0625) + 1.9 \log 2 \\ &= \bar{2}.7959 + 0.5720 = \bar{1}.3679 \\ \text{actual LD50} &= 0.233 \text{ mg.}\end{aligned}$$

Irwin and Cheeseman now proceed to find the standard error of the LD50 as follows:

Start by smoothing the observed mortalities. Fitting to a straight line will be satisfactory, although the true curve is sigmoid. Assume as before that the dose previous to the lowest gives zero mortality and stop again at the first 100% beyond any reversals. We have to fit a straight line to 0, 0.2, 0.4, 0.2, 1.0, 0.8, 1.0. This is done as follows:

(*a*) Write the mortalities (p) in a column:

	Observed p	*Cumulative p*	*Smoothed p*
	0	0	0
	0.2	0.2	6/35
	0.4	0.6	12/35
	0.2	0.8	18/35
	1.0	1.8	24/35
	0.8	2.6	30/35
	1.0	3.6	36/35
Totals	3.6 (= S_1)	9.6 (= S_2)	

and add them. Call this sum S_1.

(*b*) Take the successive sums of the items of the first column, and add these (second column). Call this sum S_2.

(*c*) Calculate (where n is the number of group mortalities used): $a = (1/n)S_1$, $b = [2/n(n + 1)]S_2$, $b' = a - b$, $Y_1 = a + 3b'$, $\Delta Y_1 = [-6/(n - 1)]b'$. Then Y_1 is the last smoothed value and ΔY_1 is the constant difference of the smoothed value. The other smoothed values are then calculated by successively adding ΔY_1. In the present case, $a = 3.6/7 = 18/35$, $b = (2 \times 9.6)/56$, $b' = 6/35$, $Y_1 = 18/35 + 18/35 = 36/35$, $\Delta Y_1 = (-6/6)\ (6/35) = -6/35$.

The last smoothed value works out to be slightly greater than one, illustrating the approximate nature of the method. It may be counted as one in the subsequent calculations. Similarly the first smoothed value may work out as slightly negative, and may be counted as zero.

(*d*) Calculate $\sigma_p^2 = \Sigma(\text{pq}/n)$ as follows ($q = 1 - p$):

	$35p$	$35q$	35^2pq
	6	29	174
	12	23	276
	18	17	306
	24	11	264
	30	5	150
Total			1170 [$= 35^2\Sigma(pq)$]

$$\sigma_p^2 = \frac{1170}{5 \times (35)^2} = 0.1910,\ \sigma_p = 0.437$$

Therefore, the standard error (σ_1) of log LD50 = 0.437 log 2 = 0.132. The value of log LD50, as computed above, was $\bar{1}.3679$. This of course is a negative number. So we have log LD50 = − 0.6321 ± 0.132.

We know from the theory of probability that 95% of results should fall within the limits of ±1.96 standard errors, and 99% between ±2.576 standard errors. Since

$$\begin{aligned}\text{antilog } (1.96 \times 0.132) &= \text{antilog } (0.2587) = 1.814, \text{ and} \\ \text{antilog } (2.576 \times 0.132) &= \text{antilog } (0.3400) = 2.188\end{aligned}$$

we may state that the probability is 95% that the true value of LD50 lies between 1.814 times the present value and the present value divided by 1.814— in other words, between 1.814 (LD50) and $\frac{\text{LD50}}{1.814}$. Thus our limits of error are 55–181%; similarly the probability is 99% that the limits of error are 46–219% of our estimated LD50.

It should be noted that the use of common fractions, so convenient in this particular illustration, is not suitable in certain other cases which may come up.

Accuracy of Comparisons of Potency

We are perhaps rather more likely to be interested in comparing the relative potencies of two or more preparations than in estimating the accuracy of the 50% end point of a single preparation. If we are comparing a new toxin, for instance, with a standard preparation, we want an estimate of how much stronger the new toxin is in terms of the standard, and an estimate of the uncertainty of this comparison. We have stated above that if we found an LD50 of 0.27 mg. for one toxic preparation, and 0.13 for another, we should say that the first was approximately half as strong as the other. We have seen illustrations of two methods of estimating the LD50. The second of these allows us to estimate the standard error of each LD50. If we knew σ exactly (instead of having only an estimate of it) we could calculate the uncertainty of the ratio of the two LD50 values. As it is, we might attempt such a calculation from the estimated standard errors, using the relation $\sigma_D = \sqrt{\sigma_1^2 + \sigma_2^2}$. Suppose we have the following two values:

Preparation	log LD50	σ_e
A	1.127	0.105
B	1.669	0.129

The difference = 0.542, and $\sigma_D = \sqrt{0.105^2 + 0.129^2} = 0.166$.

The antilog of 0.542 is 3.48. Therefore we should estimate that preparation A is 3.48 times as strong as B. The uncertainty is expressed by calculating as before,

$$\text{antilog } (1.96 \times 0.166) = 2.11$$

The chances are 95 out of 100 that the true ratio of potencies is between 47 and 211% of our estimate, that is, that the true ratio of potencies lies between 1.65 and 7.35. This is sufficient to illustrate the high degree of uncertainty which may attend comparisons of potency with small numbers of experimental animals.

When we wish to compare potencies in an actual case, it is advisable to do the calculations in a somewhat different manner from this, using methods specifically designed for such problems. This unfortunately involves statistical analysis which is rather more involved than that already given, but the general principles can be indicated. Those wishing to make actual application of the methods will find details in the references given.

If mortality rate is plotted against dosage, an asymmetrical sigmoid curve is usually obtained (see Table LXI and Fig. 49). It is not easy to draw accurately such a curve through a series of scattered points, and the resulting curves are not satisfactory for quantitative comparison. It has been found possible to reduce most such curves to straight lines by transforming the data, by substituting for the dosage units their logarithms and for the observed percentage mortality the equivalent deviations from the normal curve of error (4, 34). It is convenient to use a slight modification of the normal equivalent deviations known as probits. A straight line can be fitted much more easily to the transformed points and tables are available for making this transformation (33). From the transformed values it is possible to calculate the relative potencies of the two preparations being tested, to test whether the observed difference is significant, and estimate the precision with which the experiment has measured the difference. Since an accurate knowledge of how much we are likely to be in error may save much time in such work, and in addition may in some cases be of considerable financial significance, workers who have such comparisons to make cannot afford to ignore the modern statistical methods which have been developed to treat them. Descriptions and references will be found in the above-mentioned works and in Bliss (5, 6), Fisher (31), and Prigge (100, 101).

In a book of this character, it does not seem proper to present a detailed description of such methods. We may, however, give an example of their application to an actual case.

Lockhart (76) recorded the mortality in mice produced by different doses of *Salmonella typhimurium* "Ellinger" and "A52." His results are presented in Table LXII. It is clear that the Ellinger strain is more virulent than the A52 strain. The question is, can we measure the relative virulence, and express it in a numerical ratio? Topley and Wilson (117), who refer to these data, state that we cannot. Bliss (personal communication), however, points out that by use of the modern statistical methods such as those referred to above, we can. His calculations, which are reproduced here (Table LXII), show that the ratio $M = 1.47 \pm 0.55$, which is definitely significant. In terms of actual ratios, instead of logarithms, the Ellinger strain is 30 times as virulent as the A52.

TABLE LXI

MORTALITY OF MICE PRODUCED BY INTRAPERITONEAL INJECTIONS OF VARIOUS DOSES OF *Salmonella typhimurium*

Dose	Number of mice in each group	Mortality per cent Strain A52	Mortality per cent Strain Ellinger
10,000,000	25	92	96
100,000	25	48	88
1,000	25	44	56
10	25	16	24

It is true that the uncertainty in this estimate is great (the 95% probability limits are from 2.5 to 350), but this is because of the very flat slope of the dosage–mortality curve. Bliss points out that anyone doing new experiments with such material would probably find it advantageous to see if some other method of conducting the test might lead to a marked steepening of the curve. That need not concern us here; we wish only to point out that methods do exist for treating problems of this kind. Further details may be found in

Miller *et al.* (87). Multigraphed tables for computing dosage–mortality curves may be had free from the Division of Pharmacology, Food and Drug Administration, Washington, D. C.

"Graded Response"

So far we have been talking as if only one of two things could happen to an animal in an experiment; as if he could only die, or survive. Clearly this is a gross oversimplification. If we are assaying the potency of a preparation of insulin, for instance, we do not need to kill any of the animals. We may inject increasing doses of the preparation into various animals, and measure the fall in blood sugar which results. The degree of lowering of the sugar level will depend on the dose, with some random variation. From such measurements we may determine the relative potency of two preparations, or measure one in terms of a standard. The calculations involved are a little more complex than those we have just been through, but the fundamental statistical methods are the same. For illustrations, the reader may consult Bliss and Marks (8, 10) and Irwin (63).

Many experiments in immunology are of the all-or-none type, that is, where we do record only the death or survival of the animal. But there are often grades of response which might be recorded. For instance, in standardizing diphtheria toxin, we might take account of the length of time elapsing before death of each animal, of the severity of the symptoms, and of the findings at autopsy. Workers with diphtheria toxin have long been accustomed to lay some weight on one of more of these secondary features. In other words, they have really been treating the test, not as all-or-none, but as a procedure giving graded responses. By using a systematic scoring system and modern statistical methods, the use of graded responses can be made systematic and reliable, and each experiment can be planned so as to yield the maximum of information for the minimum expenditure of materials, animals, and labor. Since it will be found in most laboratories that these latter items are harder to obtain than a person with a working knowledge of statistics, the principles of good scientific planning would seem to call for more use of the latter. Further discussion of these points will be found in sources such as (10, 11, 31, 32, 114).

The general questions of methods of biological assay have been reviewed by Bliss and Cattell (9).

TABLE LXII

COMPARISON OF VIRULENCE OF TWO STRAINS OF BACTERIA (TABLE LXI) BY METHOD OF PROBITS (BLISS)

Strain of bacteria	Observations		Computation of provisional curves			Computation of corrected curves				
	Log dose	Per cent dead	Empirical probit	Coefficient		Expected probit	Corrected probit			
	x		Y'	X	XY'	Y	y	w	wx	wy
A 52 (S)	1	16	4.01	−3	−12.03	3.88	4.01	9.9	9.9	39.699
	3	44	4.85	−1	− 4.85	4.66	4.85	15.3	45.9	74.205
	5	48	4.95	1	4.95	5.45	4.91	14.8	74.0	72.668
	7	92	6.41	3	19.23	6.23	6.41	9.0	63.0	57.690
Total	16		20.22	0	7.30			49.0	192.8	244.262
Ellinger (U)	1	24	4.29	−3	−12.87	4.41	4.30	14.0	14.0	60.200
	3	56	5.15	−1	− 5.15	5.20	5.15	15.7	47.1	80.855
	5	88	6.18	1	6.18	5.99	6.16	11.0	55.0	67.760
	7	96	6.75	3	20.25	6.77	6.75	4.7	32.9	31.725
Total	16		22.37	0	8.41			45.4	149.0	240.540

Continued on facing page

PROVISIONAL CURVES

Combined slope: $b_c = \dfrac{2\Sigma(XY')}{IN\Sigma(X^2)} = \dfrac{2(7.30 + 8.41)}{(2)(2)(20)} = 0.393.$

Means: $\overline{Y'}_s = 5.055,\ \overline{Y'}_u = 5.592,\ \bar{x} = 4.$

Expected probits: $Y_s = 5.055 + 0.393\ (x - 4);\ Y_u = 5.592 + .393\ (x - 4).$

CALCULATION OF CORRECTED CURVES AND OF RELATIVE POTENCY

	S	U	S + U
$\bar{x}$	3.934694	3.281938	
$\bar{y}$	4.984939	5.298238	
$[wx^2]$	199.9910	171.5912	371.5822
$[wxy]$	68.3878	74.2026	142.5904
b	0.34195	0.43244	0.38374
$[wy^2]$	28.049	32.370	60.419
χ^2	4.663	0.282	5.702, $n = 5$

$$M = 3.9347 - 3.2819 - 2.6062(4.9849 - 5.2982) = 1.4692$$

$$s_M = 2.6062\sqrt{\frac{1}{49.0} + \frac{1}{45.4} + \frac{(4.985 - 5.298)^2 2.6062}{142.59}} = 0.5481$$

Relative potency = antilog $M = 29.5$, determined within limits of 2.5 and 350 at $P = 0.95$.

In this table I stands for the interval in logarithms between successive doses, and N, which ordinarily stands for the number of animals in a group, refers in this case (each probit being taken as one) to the fact that there is one value on each dose of the standard (S) and one on each dose of the "unknown" (U), and thus equals 2. The Greek letter Σ has been used here as the sign for summation, instead of the S employed by Bliss and others (see 31, 32). The meaning of the other symbols will be clear from the table itself.

4. Titration of Antitoxins

The above experimental methods could be applied to the titration of antitoxins, employing varying doses of the antitoxin and a standard lethal dose of the toxin. The remarks as to numbers of animals needed and accuracy of comparisons of potency then apply without essential change. Unfortunately, it does not seem that present practice always makes full use of the power of modern statistical methods, and many titrations of antitoxins seem to be carried out with small numbers of animals, with no attempt to estimate the error of the calculated potency.

As an unfortunate example, we may consider the methods used at the National Institute of Health (93) for testing of commercial diphtheria and tetanus antitoxins. According to this procedure, two different dilutions are made of the antitoxin, one such that one cubic centimeter should contain one unit according to the claims of the manufacturer, and one such that one cubic centimeter contains one unit, calculated to include excess to allow for deterioration with time. One guinea pig (weight as nearly as possible 250 g.) is injected with an L_+ dose of standard toxin mixed with one unit of the antitoxin according to claims, two others with an L_+ dose of toxin mixed with one unit according to the required excess. Four other animals are injected with an L_+ dose of toxin and one unit of standard diphtheria antitoxin.

The results are interpreted as follows: The dilution representing the antitoxin with its required excess of unitage in accord with the expiration date, contains a smaller absolute quantity of antitoxin than the dilution which accords with the claimed unitage. Thus for example, the dilution in accord with claimed unitage is 1:1200 and that in accord with the required excess 1:1560. The second of these dilutions should have less protective effect than the first. The animals receiving this second dose should therefore die sooner than those receiving the first. If the excess unitage be in accord with requirements, this second dose should contain one unit of antitoxin, and since the L_+ dose of toxin is used, the animals should die about the fourth day. If this smaller actual dose of antitoxin protects until the fourth day, the larger actual dose should protect at least as long (usually longer), and the animal receiving it may survive the experiment. Two animals receive the smaller dose of antitoxin and should survive at least as long as the controls, usually until the fourth day. One animal receives the large dose and, depending upon the amount in excess, may die on the fourth day, or a later day, or survive. The four control animals with the standard antitoxin unit should die upon the fourth day. No animal of the test series should die before the controls.

It may be seen that this is essentially a method of making use of graded responses, but in a somewhat unsystematic way. However, while the procedure could probably be improved, it is likely that as a rule determinations of potency made by this technic are sufficiently accurate for practical purposes.

The statistical theory of antitoxin titrations has been discussed by Trevan (118). See page 408.

5. Design of Experiments

Before any experiment is carried out, a number of points should be considered carefully. The experiment should promise to give useful information, bearing on a problem which has some importance, or special interest for the experimenter. All possible outcomes of the experiment should be considered, and the possible significance, if any, of each. Thought should be given to the question of whether or not the experiment really bears on the question which the experimenter wishes to test. It is in many cases worth while to ask oneself whether the hypothesis in question is really capable of being proved or disproved experimentally. If it is not, then it is not really a scientific hypothesis at all, and time will be saved by simply ignoring it.

Even if an experiment will clearly furnish valuable information when carried out, there is still the possibility that of the several possible ways of doing it, some will furnish appreciably more information than others, or will be definitely more economical of materials, experimental animals, or the experimenter's time. The reader will find problems of this sort very well treated in Fisher's book, *The Design of Experiments* (31), in papers by Bliss (5, 7), and in some of the above references.

In particular, if a number of different treatments are to be given a group, or several groups of animals, it is important to make sure that the different treatments are applied in a manner which is properly "randomized" (see above). By this means much uncertainty in the final interpretation can be avoided.

G. Clinical Technic

Certain general considerations governing the practical use of immunizing procedures have been discussed in Chapter X.

Whether or not immunological procedures find important application in diagnosis depends also partly on whether antibodies capable of easy demonstration are produced in considerable amount, and on how early they appear. It also depends, naturally, on how much difficulty we experience in making a diagnosis by other means, including clinical and bacteriological examination. The type of tissue response of the host is also important; for example, in diseases in which the reaction called allergy of infection (see Chapter IX) is produced, this fact is often utilized in diagnosis.

Skin tests for immunity seem to be confined to two sorts of diseases, those in which soluble toxin is an important part of the aggressive action of the microorganism, and those in which allergy of infection follows an attack of the disease or a successful immunization against it. The mechanism of the reaction obtained in the two sorts of immunity is of course entirely different (see Chapter X).

1. Injections

A number of immunological procedures require injection of material subcutaneously, intracutaneously, intramuscularly, intravenously, or intrathecally.

Vaccines and toxoids are usually administered subcutaneously, sometimes intracutaneously (vaccination against smallpox is generally performed in a somewhat special manner, described below). Serum should be given intravenously (or in a few cases, perhaps, intrathecally) whenever time is an important factor, since the concentration of antibody in the patient's circulation following subcutaneous or intramuscular injection, which may otherwise be employed, does not reach its maximum until 48–72 hours have elapsed. Intracutaneous injection is practiced in such procedures as the Schick and Dick tests, described below. In the practice of allergy a number of methods of bringing material into contact with or into the skin are in use, and are also described below.

Asepsis. The most scrupulous aseptic precautions must be observed in all these procedures. Materials injected must be sterile, the site of injection must be suitably cleansed, and syringes and needles must be sterilized, either by dry heat, boiling, or autoclaving (the hot air oven 160–170°C. for 90 minutes, boiling for 20 minutes, or autoclaving at 15 lb. for 30 minutes). Sterilization of syringes and needles by boiling seems satisfactory in practice; it is preferable to autoclave nearly all other apparatus; glassware is usually sterilized in the oven.

The importance of proper aseptic technic cannot be overemphasized; (for comments see reference 133, page 644). In particular the practice of attempting to sterilize instruments and syringes with alcohol is to be strongly discouraged.

Subcutaneous injections are commonly made in the upper arm near the insertion of the deltoid muscle. A suitable disinfecting agent for the skin consists in a 0.4% solution of sodium mercuric iodide in acetone, colored by the addition of a small amount of a dye such as erythrosin so that the disinfected site remains visible after the solvent has dried. Hypodermic needles of gage 25–26 may be used (three-quarter-inch). If the operator must give many injections per day, platinum needles offer a distinct advantage in that they may be sterilized, while still on the syringe, by heating the tip to redness in a flame. The joint between the hub of the needle and the tip of the syringe should be absolutely airtight. After the syringe is filled, it is held with the needle pointing upwards and the plunger pressed to expel air.

After inserting the needle beneath the skin, draw back on the plunger to observe if blood comes into the syringe. If it does, the needle is probably in a vein, and the needle should be withdrawn and reinserted before making the injection, in order not to make an intravenous injection by error.

When it is desired to administer large quantities of serum, the injection may be made under the skin of the abdomen.

Intracutaneous injections are described below, under the tuberculin test.

Intramuscular injections are commonly given in the upper outer quadrant of the buttocks, using a needle about 1.5 inches long, about 20–21 gage. Again it is necessary to ascertain that the tip of the needle is not in a blood vessel before making the injection. For a description and picture see Schamberg and Wright (109).

Intravenous injections are probably most suitable for the administration of therapeutic serum, particularly when time is an important factor. *They are also potentially dangerous.* It is customary to employ the veins on the inner surface of the arm at the elbow, namely the cephalic or basilic or some of their branches, although other accessible veins can be used. The skin is usually prepared by painting with iodine (unless the patient is sensitive to iodine, when some other disinfectant—not phenol—may be substituted). The iodine is washed off with alcohol. A rubber tourniquet, such as a piece of garter elastic or soft rubber catheter, or laboratory rubber tubing of the smaller sizes, is put around the upper arm, and tightened sufficiently to obstruct the venous but not the arterial circulation. The patient is requested to clench his fist to assist distension of the veins, and the needle, attached to the syringe filled with the material to be injected, is inserted, bevel edge up, into the skin over the vein, at a slight angle. Needles of about 20 gage, about 1.5 inches long, are suitable. The point of the needle is now tipped downwards directly over the vein, and pushed, by exerting a slight pressure against the elastic wall of the vein, into the lumen. Blood will usually begin to flow into the syringe, or if it does not, slight backward traction on the plunger will cause it to do so. Once it is certain that the point of the needle is in the lumen of the vein, the syringe is lowered until parallel to the vein, and advanced so as to bring the point far enough in to prevent its slipping out. Backward traction must again be used to make sure that the point is still within the lumen. Then the tourniquet may be released and the injection slowly made. Descriptions of the technic will be found in Dutton (28) and Schamberg and Wright (109).

Precautions. Before injection of serum or any material to which the patient may be sensitive, and especially before intravenous injections, it is necessary to take certain precautions.

A 1:1000 solution of adrenaline (epinephrine) and a sterile syringe and needle should always be ready for immediate use, and if symptoms such as asthmatic wheezing, marked cyanosis, a sense of substernal constriction and choking, or "collapse" appear, 0.5 to 1.0 cc. of the adrenalin should be given subcutaneously or, if the reaction is severe, intravenously. In the latter case only about one-third of the adrenaline is given at once, and the remainder introduced slowly, the patient being observed during the process. In the severest cases, amounts up to two to four cubic centimeters may be needed, but should not be injected too rapidly.

Before serum is injected, the patient should be questioned carefully as to previous history of sensitiveness, and a skin test made for sensitivity to the kind of serum (e.g., horse or rabbit) being used. Detailed inquiry should be made as to any personal or familial allergic symptoms, including asthma, hay fever, food or drug sensitiveness, urticaria, or angioneurotic edema. The patient should be asked about any previous injections of serum, referring particularly to diphtheria immunization with toxin–antitoxin, tetanus prophylaxis with serum, and treatment for diphtheria or pneumonia.

An intracutaneous test is made, using 0.05 cc. of a 1:10 dilution of the serum. Some consider it preferable to use normal serum (from the species furnishing the immune serum it is proposed to use). A positive test consists of a typical urticarial wheal with surrounding erythema. It should reach its maximum within 30 minutes. Adrenaline should be at hand against the possibility of extremely sensitive individuals being found.

Some perform a conjunctival ("eye") test also, with the same material used for the intracutaneous test. A single drop placed in one eye is sufficient. The other eye serves as a control. If the test is going to be positive, the reaction occurs within five to ten minutes. It is characterized by redness (due to injection of the conjunctival vessels), itching, and lacrimation. A good picture in color is shown in Tuft (119). The reaction ordinarily disappears after a few hours, but after a marked reaction, swelling of the conjunctiva or the lower lid may persist from 24 to 48 hours. Excessive discomfort may be controlled by the use of adrenaline (1:1000) in the eye. Usually a positive eye reaction indicates a much higher degree of sensitivity than does a positive skin test alone, and is thus to be considered as a sign of potential trouble if serum is given.

If no reaction is obtained in the test, the injection may be given with fair expectation of no severe reaction following, although this assurance is not absolute. If serum must be given to individuals showing a positive test, and an equivalent antiserum from a different species is not available, the greatest caution should be used, and it may be well to attempt a specific desensitization (see page 325) first.

The pulse rate and rhythm and blood pressure should be noted before each injection, and observed afterward. A pulse of 140 or over, an irregular pulse, or falling blood pressure are indications to proceed with caution, *or to stop*.

The serum should be warmed to body temperature by immersing the container in warm water at about 110°F., but should not be held under the hot water tap. No solid matter or coagulum should be present in the serum. The first injection is begun by allowing the smallest possible amount to enter the vein, and watching the patient's reaction for five minutes with the needle still in place. This is then repeated with a few tenths of a cubic centimeter of serum. In the absence of reaction the injection can proceed slowly, so as to be completed during the next five or ten minutes. Subsequently the patient should be observed for at least 15 minutes, or, much better, 45 minutes. A good discussion of the procedure of injecting serum is given by Finland (30).

Intrathecal (intraspinal) injections of serum are sometimes recommended. This operation should be undertaken only by a person with considerable experience, and it should be learned by actual demonstrations and practice. It is doubtful if a description of the technic would be of any particular value here. We may mention, however, the need to govern the amount of serum injected by the amounts of fluid withdrawn.

2. Diagnostic Tests

Tuberculin Tests

The intracutaneous (Mantoux) test will be described. Old tuberculin (OT) was formerly used for this test, but it is likely that the purified protein derivative of Seibert (PPD) will replace it, for it enables a definite amount of purified tubercular antigen to be employed for each test, and seems to be somewhat more specific (46, 113).

One-tenth of a cubic centimeter of either a 1:1000 dilution of OT, or 0.1 cc. of a dilution of PPD containing 0.0002 mg. per cc., is injected intracutaneously. A weaker dilution, e.g., 1:10,000 of OT is used for patients suspected of having active pulmonary, bone and joint, skin, or opthalmic types of tuberculosis. If the reaction is negative, the test is repeated with the same amount of OT diluted 1:100, or a solution of PPD containing 0.05 mg. per cc.

The skin of the forearm is washed with alcohol and allowed to dry. Then 0.1 cc. of the proper dilution of the material is injected into the skin of the flexor surface of the forearm, 4–5 cm. below the bend of the elbow, using a sterile 1 cc. tuberculin type syringe and a 26-gage needle one-fourth to one-half inch in length. The bevel of the needle is held uppermost and the needle inserted parallel to the skin, just covering the bevel, so that material does not leak out as the injection is being made, but the puncture does not extend beneath the skin. A sharply defined, more or less hemispherical swelling, raised about 1 mm. above the surrounding skin, results.

The reactions are read 24 and 48 hours later. A positive reaction shows erythema and edematous infiltration with a diameter greater than five millimeters. Larger reactions may be graded as more severe. For references, see (112, 113, 119).

The diagnostic significance of the tuberculin test is not easy to summarize. A positive reaction simply shows allergy to tubercle protein, indicating a tubercular infection which may be small or extensive, active or healed. A negative test with a strong solution, say a 1:100 or 1:10 dilution of OT, or 0.05 mg. PPD practically excludes the existence of tuberculosis infection (see 43). A positive reaction is more likely to be associated with active infection in infants and young children than in adults (38, 46); since a large proportion of adults have at some time been infected with tubercle bacilli, a positive test with them is of less significance. Some workers have suggested that the adult with a positive tuberculin reaction is on the whole somewhat more resistant to serious tuberculous infection (compare Chapter IX).

3. Tests for Immunity

Schick Test

If diphtheria toxin is injected in proper amount into the skin, it will be neutralized in individuals who possess antitoxin, while in susceptible individuals without antitoxin it will produce an imflammatory reaction. This is the basis of the Schick test (86, 110). The procedure is described in (108).

The material formerly used consisted of one solution containing $\frac{1}{50}$ MLD of aged (i.e., relatively stable) diphtheria toxin in 0.1 cc., and another solution which consisted of toxin which had been heated to abolish toxicity. The latter solution serves as a control on allergic reactions to other components of the toxic filtrate, usually to thermostable proteins of the diphtheria bacillus. Taylor and Moloney (116) proposed a Schick toxin made from "fresh toxin," containing $\frac{1}{50}$ to $\frac{1}{35}$ MLD in 0.1 cc., with somewhat less antitoxin combining power (1 dose neutralized 1/1250 but not 1/1500 unit of antitoxin). This seems to be better (21).

Cleanse the skin of the flexor surface of both forearms with alcohol or acetone and allow to dry. On the right arm inject intracutaneously 0.1 cc. of the heated toxin dilution (control); on the left arm inject intracutaneously 0.1 cc. of the toxin dilution. The injection technic is the same as that for the tuberculin reaction, above.

Read the reactions on the first or second *and* on the fourth day following the test. If the test has been properly carried out, and the toxin is of the correct potency, absence of a reaction indicates immunity to diphtheria. A positive reaction usually begins to appear in 18–24 hours, and presents a circumscribed area of redness and slight infiltration 1–2 cm. or more in diameter. It reaches its greatest intensity on the fourth day and gradually fades. More or less desquamation follows and an area of brownish pigmentation, more marked in brunettes, may remain for weeks or months. Vesiculation occurs in very intense reactions, which indicates virtually complete absence of antitoxin in the subject tested. The degree of reaction is a very roughly quantitative measure of antitoxin. A negative reaction was formerly taken to indicate the presence of at least 0.03 unit of antitoxin per cubic centimeter of serum; more recent estimates have reduced this figure to about 0.004 to 0.01 unit.

In some individuals, especially in adults, an allergic reaction to bacterial proteins, called the "pseudo reaction," may develop. It develops and evolves somewhat more rapidly than the true positive reaction, so that by comparing the rate of development and size of the reactions on the two arms, the combined reaction, where susceptibility to toxin and bacterial allergy are both present, can be differentiated from the pseudo reaction. In the latter the reactions on both arms run an equal course, reach their height in about 48 hours, and are of nearly the same size.

In highly allergic subjects it may be difficult or impossible to distinguish between pseudo and "combined" reactions even at the fourth-day reading. In such cases the final reading may be delayed until the seventh to tenth day when desquamation and discoloration are more marked on the test site than on the control site.

It is important, when dealing with older children and especially with adults, that the early reading be made in order to detect the allergic reactions. Combined reactors are liable to develop severe local and systemic symptoms, when actively immunized with diphtheria antigens, especially toxoid. The occurrence of an allergic reaction is a warning that the usual primary immunizing dose must be greatly reduced. The amount of allergenic material in the Schick control dose is so very small, especially when the extraordinarily potent toxin now obtainable (Mueller) is used, that only the highly allergic subjects respond to it, and the more moderately sensitive ones escape detection; they, however, can be made sick by the routine doses used in active immunization, so it is desirable to resort to the Moloney test in order to obtain a better guide to the selection of an appropriate primary immunizing dose.

The negative and pseudo reactions indicate immunity, the positive and combined reactions indicate susceptibility as shown in Table LXIII.

References and descriptions of the technic will be found in (12, 108).

Moloney Test

Moloney and Fraser (89) introduced the injection of 0.1 cc. of a 1:20 dilution of toxoid, as a substitute for the control in the Schick test, and an indicator of possible reactors to immunization; a similar test had previously been proposed by Zoeller (see 134). The test is now widely used, but the dilution of toxoid originally proposed seems much too strong; it is possible that its routine use would produce a number of severe or even constitutional reactions. Underwood (121) recommended 1:200, but it would seem that a 1:100 dilution is most generally used.

TABLE LXIII

INTERPRETATION OF REACTIONS TO THE SCHICK TEST

No.	Left arm (toxin)	Right arm (control)	Reading	Interpretation
1	Positive	Negative	Positive	NOT IMMUNE, not allergic
2	Negative	Negative	Negative	IMMUNE, not allergic
3	*Positive*, larger than (1)	(Pseudo-) positive	Combined	NOT IMMUNE, allergic
4	(Pseudo-) positive	(Pseudo-) positive	Pseudo	IMMUNE, allergic

Bunch *et al.* (19) recommend the following plan:

(*1*) Do a Schick test on the group to be immunized.

(*2*) Do a Moloney test on the Schick-positive individuals.

(*3*) Give the M− and M+ (see below) reactors the routine two cubic centimeters of undiluted toxoid in 1-cc. subcutaneous doses with an interval of one month.

(*4*) Give the M++ and M+++ reactors three 0.1-cc. intracutaneous injections of 1:100 dilution of toxoid at weekly intervals.

(*5*) Do Schick tests three to six months after the last injection.

(*6*) If any M+++ or M++ subjects remain Schick positive, inject them with a series of graded subcutaneous injections.

The test is performed by injecting intracutaneously 0.1 cc. of diphtheria toxoid diluted 1:100. The reactions are read after 24 hours and standardized (82) as follows:

No reaction = M−
Erythema less than 10 mm. without induration = M+
Erythema more than 10 mm. without induration = M++
Erythema with induration = M+++

Dick Test

This is an intracutaneous test for immunity or susceptibility to scarlet fever, quite analogous to the Schick test. The scarlatinal toxin used has been standardized by tests in the skin of susceptible persons so that one skin test dose (STD) is contained in a volume of 0.1 cc. As in the Schick test, heated toxin (100°C. for two hours) is used as a control on possible allergic reactions. Pseudo and combined reactions may occur but are very rare following primary tests and uncommon even in retests after active immunization. This allergic reaction usually lasts longer than the simple reaction to toxin.

The reactions are read after 18–24 hours. Differences in size and intensity (redness) of the reaction indicate degrees of susceptibility. A reaction is considered positive if there is an area of redness at least 1 cm. in diameter with a negative control (26).

The reliability of the Dick test does not seem to be quite as high as that of the Schick test, for Dick-negative individuals may sometimes contract scarlet fever, although only about one-twentieth as often as Dick-positive individuals (1).

4. IMMUNIZATION

Active Immunization

Antityphoid Immunization. As an example of active immunization employing vaccines consisting of killed microorganisms, we may describe the procedure for immunizing against typhoid fever.

The vaccines used in this country are prepared from the Panama 42-A-58 strain of *Eberthella typhosa*. They are standardized to include one billion (1000 million) killed organisms per cubic centimeter.

It was long the common practice to employ a mixed vaccine containing also killed paratyphoid bacilli A and B (usually 750 million per cubic centimeter of each). This has been largely discontinued in this country, because of the low incidence of paratyphoid infection.

Of course, in any locality where these or other paratyphoid types are prominent enough in producing infection, any or all could and should be introduced into the local vaccine. During the Second World War, the U. S. Army used vaccines containing paratyphoid A and B as well as typhoid (20).

It was once customary to give three subcutaneous doses, of 0.5, 1.0, and 1.0 cc., of the vaccine at intervals of seven to ten days. It has now become common to give it intracutaneously, in doses of 0.1, 0.15, and 0.2 cc. According to Tuft (120) this is the most satisfactory method of administration, mostly avoiding the systemic reactions which may follow subcutaneous injection of the vaccine, and giving a satisfactory increase of the protective power of the blood and stimulating the production of somatic agglutinins. A single subcutaneous injection of 0.1 cc. seems to be adequate to restore the immunity of those vaccinated previously (even 20 years before). An intracutaneous injection of 0.1 cc. is considered enough to keep up immunity, if repeated at one year intervals (77).

As an example of the effectiveness of antityphoid inoculation, see the statistics shown in Table LVIII.

Antidiphtheria Immunization. This may be taken as typical of the procedures employing toxin or modified toxin for immunization. The toxin–antitoxin mixtures formerly used have been displaced by toxoid (formalin-detoxified toxin). It is generally administered in three subcutaneous doses of 0.5, 1.0, and 1.0 cc. at intervals of two to three weeks. It is likely that the immunizing effect is better if the intervals are somewhat longer (three to four weeks). More than 1.0 cc. should not be injected at one time.

When immunizing adults it is often necessary, on account of the severity of the reactions, to dilute the toxoid and give a larger number of injections. Therefore, some workers still advocate the use of toxin–antitoxin for the immunization of adults. It is hard to be convinced that this would offer any advantage, since the allergenic properties of the proteins will not be diminished by the presence of the antitoxin, whereas the immunizing power may be somewhat lessened. At least as much, probably more, diphtheria protein will have to be employed, and at the same time there is introduced the unnecessary complication that sensitization of the patient to horse serum will frequently occur.

Scarlet Fever Prophylaxis. Active immunization by the injection of scarlatinal toxin has now been practiced enough, particularly in the case of nurses, internes, and others frequently exposed to scarlet fever, to make it fairly certain that immunization, carried to the point of making the Dick reaction negative, gives a considerable degree of protection against the disease (3, 25, 26, 42, 68, 132). This immunity does not seem to be permanent, and it has been estimated that it lasts from three to five years. It is likely that at the end of this time it can be fully restored by one or two further injections; that is, a repetition of the full course of injections would not be necessary.

It can easily be understood that immunization with scarlatinal toxin would probably fail to protect the individual against strains of the hemolytic streptococci other than the scarlatinal types. There seems to be some indication that this is the case. More than this, however, there is evidence that some immunized individuals may still contract throat infections due to scarlatinal streptococci (67). Benson (3) observed that the decrease in the incidence of tonsillitis among an immunized nursing staff was much less than the decrease in scarlet fever.

Since the unattenuated toxin is usually used for immunization, rather severe local reactions have sometimes been observed. Some physicians, apparently believing that all immunization, like Gaul, should be divided into three parts, attempted to complete the immunization by giving only three large doses, and obtained reactions almost as severe as the mild type of scarlet fever which prevails at the present time in this country. Even so, however, it would hardly be correct to state, as some have, that the remedy was worse than the disease, for with the real disease there is always the possibility of distressing and disabling sequelae. It is better to give more than three injections, subdividing the same total unitage correspondingly. Reactions are thus much diminished.

Hooker (private communication) uses the basic schedule shown in Table LXIV for scarlet fever immunization, but makes the actual size of the dose depend upon the degree of reaction previously obtained. Thus the first dose would be smaller in individuals who gave a large, bright red reaction to the Dick test (26), the second dose would depend upon the reaction obtained with the first, etc. A dose is given once each week for seven weeks. Two weeks later the patient is Dick tested, and if the test is not negative, another dose equivalent to the last is administered. According to Kolchin and Klein (68) the use of excessive immunizing doses is futile in persons not responding to immunization with the usual doses.

Some consider the necessity of so many injections a serious drawback, and no doubt it would tend to prevent the routine use of scarlet fever immunization, even if it were generally considered that the form of the disease at present prevailing in this country justified such routine immunization. Some patients object to having the needle applied to their arms so often, and the expense of so many visits to a physician is a factor with others. Others, however, with whom the present writer agrees, are accustomed to regard immunization as a very cheap form of insurance, well worth its cost, even though several injections are needed, as in scarlet fever. The decision in individual cases will of course depend on the attitude and resources of the patient and his physician.

The toxin used in scarlet fever immunization is commonly that produced by the Dochez strain of the scarlatinal hemolytic streptococcus, which is often referred to as N.Y. 5. Methods of producing toxin from this organism have been described by Hooker *et al.* (60). According to Zinsser, Enders, and Fothergill (134) this technic is now generally employed. Scarlatinal toxoid has been recommended by some workers for use in immunization, but it is apparently still in the trial stage.

TABLE LXIV

TENTATIVE DOSAGE SCHEDULE FOR IMMUNIZATION WITH SCARLET STREPTOCOCCUS TOXIN (HOOKER)

Injection No.	Solution No.	Dose (cc.)	Injection No.	Solution No.	Dose (cc.)
1	1	0.1	5	2	0.2
2	1	0.3	6	2	0.45
3	1	0.6	7	2	1.0
4	2	0.1			

Solution number 1 contains 5000 skin test doses (STD) per cubic centimeter, solution number 2 contains 50,000 STD per cubic centimeter.

Smallpox Vaccination. This is an example of methods of immunization by which a living virus is introduced into the patient's body, and probably the safest and most successful. However living attenuated virus has also been used with good results in the case of yellow fever (27). Also in several bacterial diseases such as plague and brucellosis the use of attenuated strains seems to have given encouraging results (27). For immunization to tuberculosis see page 373.

The best place to vaccinate right-handed persons is in the skin of the left arm, over the insertion of the deltoid muscle. The skin is washed gently with soap and water, then with alcohol or preferably acetone, and allowed to dry. Medicated alcohol, iodine or other strong nonvolatile germicides can not be used because they are likely to kill the virus. The virus should be fresh, i.e., used before the expiration date on the label, and must be kept continuously on ice before use.

A small drop of virus is placed on the cleansed area, the arm is held by the operator's left hand in such a way that the skin is pulled taut. Then a sharp sterile needle is held in the right hand parallel to the skin of the patient's arm, and the side of the point of the needle pressed firmly into the drop of virus about 30 times, the needle being lifted clear each time. The total area treated should not be over one-eighth inch in diameter, and the pressure should not be great enough to cause bleeding, which could wash out the virus, though it obviously must be sufficient to make tiny abrasions. The remaining virus is wiped off with sterile gauze. No dressing or shield should ever be used except, perhaps, light gauze for children.

The site should be examined on the first, second, and seventh day to see if the vaccination has "taken." Several different types of reaction may be obtained, depending on the degree of immunity of the patient (see page 18). The longer it takes the reaction to develop, as a rule, the more severe it will be, and the less, therefore, the previously existing immunity. In immune individuals the reaction appears rapidly and occasions no inconvenience. It is important to realize that some reaction should follow vaccination, for otherwise it has not been properly done, or the virus has lost its potency.

The various reactions have been described by Leake (72): "With a reaction of immunity which indicates full protection against smallpox, the broadest redness is reached and passed in 8 to 72 hours after vaccination. This redness is accompanied by a slight elevation of the skin, which can be felt by passing the finger lightly over the vaccinated area.

"With the accelerated or modified reaction, called vaccinoid, which indicates partial immunity, the broadest redness is reached and passed in 3 to 7 days after vaccination.

"With a typical primary vaccination or vaccinia, indicating absence of immunity to smallpox prior to this vaccination, the zone of redness, or areola, rather narrow from the third to the seventh day, begins a sudden spread about 7 days after vaccination and reaches its broadest diameter in 8 to 14 days after vaccination, rapidly disintegrating and disappearing thereafter.

"The sharp division of the post-vaccinal reaction into these three types is somewhat arbitrary, all gradations being found in practice; differentiation into the three types is based on the time of broadest redness. The more prompt the maximum the higher is the immunity. Vesicles are formed in vaccinoid and typical vaccinia reactions, but not usually with the reaction of immunity. The vesicle of a typical vaccinia, and of many vaccinoid reactions, has a turbid, whitish

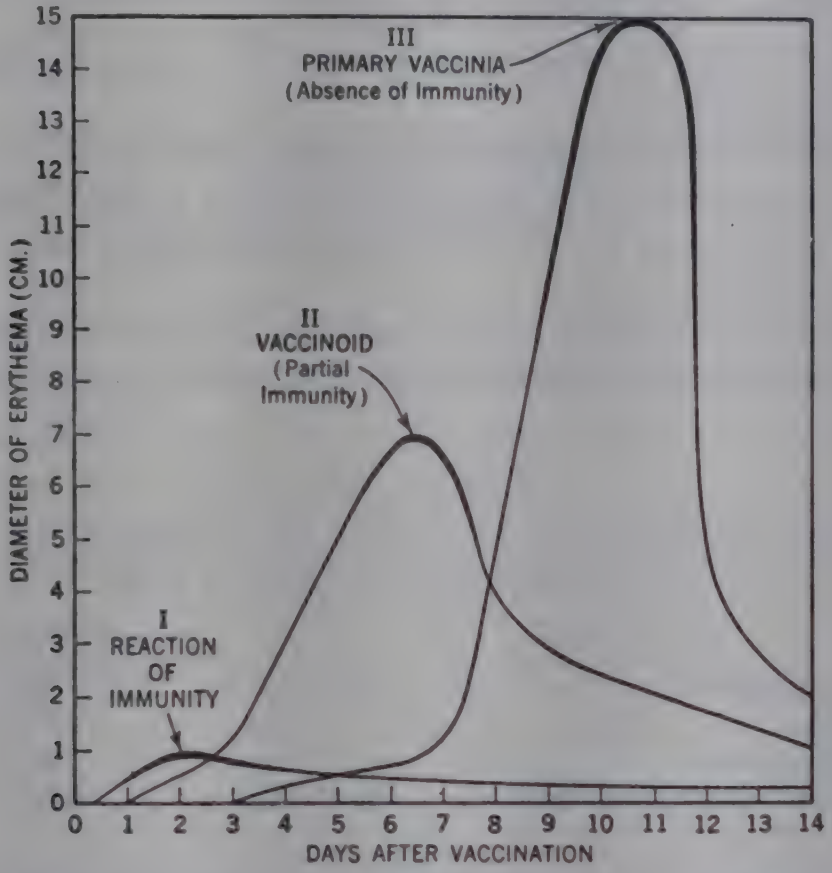

Fig. 50. Three types of reaction following vaccination, as differentiated by the diameter of the area of erythema (72). (I) *Immune reaction:* This is the reaction of a person fully protected against smallpox by previous vaccination or previous attack of smallpox. Maximum diameter of erythema reached in 8–72 hours. Usually no vesicle. (II) *Vaccinoid:* Indicates partial loss of previous immunity. Maximum diameter of erythema in 3–7 days. Vesicle at center of reaction. (III) *Primary vaccinia:* Reaction of a person previously unvaccinated, never having had smallpox, or having lost all previous immunity. Maximum diameter of erythema in 8–14 days. Vesicle at center of reaction. Varying depth of color over entire red area indicated by thickness of curves.

appearance, but if properly cared for does not become a true pustule, and dries up and heals promptly after the height of the reaction is reached. The characteristic pitted scar is red at first and gradually becomes white. Scars of vaccinoids are much less marked, and reactions of immunity usually leave no scar.

"All three of the types are successful vaccinations provided the smallpox vaccine was fresh and strong."

The relationships of these reactions are well shown by Figure 50, taken from Leake (72).

Children should be vaccinated during the first year of life, for undesirable complications are less likely to follow at that time. This should be followed by revaccination when the child is ready to start to school (44).

It is generally considered that an individual should not regard himself as completely protected unless he is vaccinated about every ten years (117). The value of vaccination in reducing smallpox in the population is shown by Table LXV.

TABLE LXV

RELATION OF SMALLPOX MORBIDITY TO VACCINATION LAWS IN THE UNITED STATES, 1919–28 (129)

Vaccination laws	Cases per 100,000 population
Compulsory vaccination	6.6
Local option	51.3
No vaccination laws	66.7
Compulsory vaccination prohibited	115.2

Passive Immunization

Against Diphtheria. This may serve as an example of methods of passive immunization by injection of antiserum. As has been previously explained, such protection is temporary, and active immunization is to be preferred, except when not enough time is available, or the incidence of the disease is so low that routine immunization would not be justified.

Individuals who have been exposed to diphtheria *may* be protected by the subcutaneous injection of 1000 units of antitoxin. The protection conferred in this way lasts only two to four weeks at most.

5. SERUM TREATMENT

Diphtheria Antitoxin

We shall discuss here two examples of the treatment of a disease with antiserum. Our first is an example of the use of an antitoxin.

In the treatment of diphtheria with antitoxin, it is important to administer an adequate dose at the earliest possible moment. The action is most rapid if the injection is given intravenously. Since serum given in this way is more likely to cause reactions, which may be severe, than intramuscular injections, some authorities prefer the latter route in mild cases where speed is not so urgent.

TABLE LXVI

UNITS OF ANTITOXIN TO BE ADMINISTERED TO CASES OF VARYING GRADES OF SEVERITY

I (after Park, 96)

Age or weight of patient	Mild cases[a]	Early moderate[b]	Late moderate and early severe[c]	Severe[c] and malignant[d]
Under 2 years, 10–30 lb. wt.	2,000–3,000	3,000– 5,000	5,000–10,000	7,500–10,000
2–15 years, 30–90 lb. wt.	3,000–4,000	4,000–10,000	10,000–15,000	10,000–20,000
Adults, 90 lb. or over	3,000–5,000	5,000–10,000	10,000–20,000	20,000–50,000
Route of administration	Intramuscular	Intravenous	Intravenous	Intravenous

II (as recommended by the Massachusetts Antitoxin and Vaccine Laboratory)

Mild cases[a]	Moderate[b]	Severe[c]	Malignant[d]
5,000–10,000	10,000–20,000	30,000–50,000	50,000–100,000

The larger amount should be used if antitoxin is given intramuscularly. Intravenous administration of part of the dose is recommended in severe and malignant cases.

Modern practice inclines to the larger doses shown in schedule II.

[a] Membrane limited to one tonsil or to the nares.

[b] Both tonsils involved or one tonsil and adjacent pillars.

[c] Extension of membrane to uvula, soft palate or nasopharynx.

[d] Extensive membrane, extreme toxicity, hemorrhages in mucous membranes, skin, etc.

The amount to be given depends on the severity of the disease, the stage at which the patient is admitted or diagnosed, and the patient's age. The dosages recommended are given in Table LXVI.

Nasal and laryngeal diphtheria, moderate cases seen late at the time of the first injection, and diphtheria occurring as a complication of the exanthemata should be classified and treated as "severe" cases (96). Park *et al.* (96) recommended that the methods of administration be as follows:

Mild cases: intramuscular
Moderate cases: intramuscular
Severe cases: intravenous, or both intramuscular and intravenous
Malignant cases: intravenous

Antipneumococcus Sera

The use of these sera furnishes us with an example of the therapeutic use of antibacterial sera.

If pneumococci are found in large numbers in characteristic rusty sputum, or in blood cultures or lung puncture material, serum of the corresponding type should be given if it is available and if the patient is still acutely ill. At the present time this is usually supplemented by chemotherapeutic treatment. According to Finland (30), experience has shown that finding types 1, 2, or 5, and probably types 7 or 8, in sputum even in small numbers, or only after mouse inoculation of throat cultures, is adequate indication for using the corresponding sera.

Antisera from the horse and from the rabbit are both available. The rabbit sera have displaced the horse sera in the hands of most physicians. Finland (30) advises against the use of type 14 horse serum (which is probably no longer available anyway) because it may cause severe or even fatal reactions because of the antibodies for human erythrocytes which it may contain.

The sera are almost always given intravenously. Finland (30) suggests that most of the available sera can be diluted with two to ten parts of sterile saline for the first injection, which must be given very slowly. In small children the diluted serum can usually be given with a syringe and a 25–26-gage needle; in the adult, if unconcentrated rabbit serum is used, and with some preparations of concentrated rabbit and horse sera, the serum may be given in a slow intravenous drip (about four cubic centimeters per minute), after dilution in about a liter of sterile saline. The usual precautions when injecting serum should be observed, and a test injection of 0.1 to 1.0 cc., diluted with 5 to 10 cc. of saline, should be given first, followed by at least 20 minutes of observation.

6. ALLERGY

Skin Tests

These tests form an important part of methods of diagnosis of allergic conditions (see Chapter IX). Patients should not be skin tested without previous adequate history and physical examination to detect other causes of their symptoms, however.

The allergens to be used for the tests depend upon the history, symptoms, the age of the patient (thus an infant can hardly have food allergy to more than the limited number of substances comprising his diet). The technic of making the test depends to some extent upon the preference of the operator.

The Intracutaneous Test is performed on the skin of the forearm or the skin of the outer aspect of the arm, or the skin of the back may be used. The skin is cleansed with alcohol. A very small amount (0.01 to 0.02 cc.) of the sterile diluted extract is injected with a tuberculin syringe into the outer layer of the skin. The technic of intracutaneous injection has already been described above under the tuberculin test. The reactions are usually fully developed in from 5 to 15 minutes; they may last varying lengths of time, up to many hours. A control consisting of the diluent should be injected also.

The Scratch Test. This is performed upon the skin of the forearm or of the inner aspect of the arm. The skin is first cleansed with alcohol and dried. A small superficial scratch about one-eighth inch long is then made through the epidermis with a small sterile needle or other suitable instrument. There should be no bleeding. If several tests are made at once, all the scratches are made first, at least one or one and one-half inches apart, and one is kept as a control. Liquid allergens may be applied directly to the scratch; if a powder is used, a drop of diluent (0.01 *N* sodium hydroxide* or saline) is first applied, then enough powder to cover the end of a toothpick is mixed with the diluent, using the toothpick. The reaction usually reaches a maximum in from 15 to 30 minutes. The material is wiped off and the reaction read. If the reaction appears sooner, as it may in highly sensitive individuals, the material is wiped off at that time.

A positive reaction in either of the above tests consists of a whitened wheal (larger than the control), surrounded by an area of redness. In the more marked reactions the wheal may develop irregular projections called pseudopodia, and the area of redness may be quite large. The degree of itching also helps in grading the degree of reaction.

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* Very irritating to some skins.

The intracutaneous test is from 50 to 1000 times as delicate as the scratch test. Its relative advantage is somewhat lessened, however, by the fact that false (nonspecific) reactions are more common. In particular the hard, round nonpseudopodial "button" reactions following the intracutaneous injection may be nonspecific.

The Patch Test finds useful application in the allergic contact dermatoses. The suspected agent is applied to the cleansed skin by means of a half-inch square of clean linen or blotting paper, which has been soaked in the allergen if it is a liquid, covered with it if it is an ointment, or moistened and sprinkled with it if it is a powder. Pieces of suspected clothing, etc., may be applied directly. The square is now covered by a larger piece of rubber tissue, oiled silk, or cellophane, which is in turn covered by a piece of adhesive one to two inches square. Unless the patch proves too irritating, it is allowed to remain in place for 24 hours; sometimes 48–72 hours is necessary. The site is inspected when the patch is removed, and if negative, daily for the next few days and at intervals for the next two weeks. An erythematous reaction at the site, which may contain vesicles, constitutes a positive reaction.

The Indirect (Prausnitz-Küstner) Test. This type of test is valuable for patients with atopic dermatitis (eczema) who are difficult or impossible to diagnose by direct skin tests. The reaction depends upon the presence of sensitizing antibodies (reagins) in the patient's blood, but it must be remembered that the absence of reagin does not necessarily show the absence of sensitivity to the allergens used. A subject should be used who is not allergic and whose skin is receptive (see page 329).

A small amount (0.05 to 0.1 cc.) of the patient's serum is injected intracutaneously into the subject's skin, at as many sites as it is desired to test. After an interval of 72–96 hours, during which time, if the suspected allergens are certain foods, the subject refrains from eating them, a small amount (0.02 cc.) of the suspected excitant is injected into one of the sites (which may be marked by circling with ink) and into an adjacent unsensitized area of skin for a control. If a positive ("immediate") reaction is obtained in the sensitized site, and the control is negative, this indicates the presence of sensitizing antibody in the blood of the patient. Any definite difference between the reaction of the prepared area and the control is considered as possibly significant.

The technic of all these skin tests may be found, together with excellent pictures, in Tuft (119).

Specific Desensitization

This is a potentially dangerous procedure, and should not be attempted without expert guidance or some experience. The initial dose should be judged from the intensity of the cutaneous reaction, and adrenaline should be at hand at all times. Tuft recommends that if the test were done by the scratch method, the initial dose should be 0.1 cc. of the strongest dilution which fails to give a positive reaction; with the intracutaneous test, the average first dose should be 0.1 cc. of the highest dilution giving a moderate reaction. Subsequent doses are increased in accordance with the patient's reaction to previous doses. It is generally attempted to reach ultimately a dose of 100 to 1000 times the size of the initial dose, making use of increasing concentrations to keep the actual volume injected within practicable limits, but there is great individual variation from patient to patient.

The interval allowed to elapse between doses depends upon the allergen and the type of allergic disease. In the treatment of hay fever the pollen extract may be injected daily, weekly, or monthly, depending upon the patient and the type of procedure decided upon. Allergens are usually injected at intervals of four to seven days (119). As desensitization progresses, the intervals may be gradually lengthened to two, three, or four weeks. The injections are given subcutaneously.

SUMMARY

In this final chapter, certain aspects of practical and laboratory use of immunological methods have been discussed. Even in a relatively long chapter, by no means all important experimental technics could be included, so the material presented is in many respects a somewhat arbitrarily chosen sample of what is available. More information may be gained by consulting the references.

In spite of the inevitable gaps in the material presented, however, the chapter probably includes more material than will be covered in the average practical course in immunology. Therefore a summary such as those given at the end of Chapters I to X inclusive would be of little value, in the absence of any knowledge as to exactly which portions of the chapter will be assigned in any given course. The student is therefore invited to make his own summary of the portions studied by him, perhaps following the model of the summaries of the preceding chapters. A list of the material presented will be found in the Table of Contents at the beginning of the book.

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Chapter XI

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